A Dissertation

for the Degree of Doctor of Philosophy

Comparative Genomic Analysis of Probiotic Properties of Host-Adapted *Lactobacillus* spp. Isolated from Pigs

돼지 유래의 숙주 적응 락토바실러스 균주들의

생균제 특성에 관한 비교유전체학적 연구

August, 2017

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농 학 박 사 학 위 논 문

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이 논문을 농학 박사 학위 논문으로 제출함.

2017 년 5 월

서울대학교 대학원 농생명공학부

이 준 영

이준영의 농학 박사학위논문을 인준함.

2017 년 7 월

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Summary

Although probiotics are regarded as AGP alternatives, unproven probiotic strains are frequently used in the animal and functional food industry. Thus it is necessary to understand exact functions and mechanisms of probiotic microbes, and not only health benefits to hosts but also their host adaptation properties should be considered for probiotic selection. Host-specialized microbes show host adaptation properties that were acquired during evolutionary process. Therefore, understanding direction and features of the microbial evolution is important to screen probiotics. Comparative genomic approach helps us to understand functional properties of probiotic microbes and ecology of the microorganisms. In this research, *L. salivarius* and *L. reuteri* among probiotic microbes were selected to investigate probiotic properties mentioned above. These two species are important members in the gut microbiota of vertebrates and known as promising probiotic species. Fecal samples were collected from several pig farms in Republic of Korea, and 634 LAB were isolated from the feces. Among them, 284 *L. salivarius* strains and 104 *L. reuteri* strains were identified using species-specific multiplex PCR.

In study 1, 21 strains were randomly selected among the 284 *L. salivarius* strains and the draft genomes were generated *de novo*. These strains were composed of 6 strains isolated before the AGP prohibition (SBPs) and 15 strains isolated after the AGP prohibition (SAPs) at an interval of a decade. The pan-

genome of *L. salivarius* strains isolated from humans, pigs and chickens were investigated using all available genomes obtained from NCBI genome database, focusing on host adaptation. MLSA-based phylogenetic clustering showed a distinct categorization of *L. salivarius* strains depending on their hosts. Among 4,431 orthologs in the pan-genome, 15 host-specific genes and 16 dual-host-shared genes were identified, and several genes associated with host-specific extracellular proteins were identified. 56 extracellular protein encoding genes and 124 orthologs related to EPS production in the pan-genome were also examined, and the distribution and/or nucleotide sequence of these genes are distinguished by the bacterial hosts. Furthermore, some host-specific genes are responsible for energy production and resistance against host stress. In summary, *L. salivarius* has evolved to adapt to host habitats in three ways, by gaining the abilities for niche adhesion, efficient utilization of nutrients, and resistance against host stress. Furthermore, *L. salivarius* acquired some genes that have mutualistic properties, contributing to increase the fitness of both the hosts and the microbes during the evolutionary process.

It was hypothesized that the microbial genomes have also been affected by the legal prohibition of AGPs, and *L. salivarius* was investigated in terms of influence of the AGP ban. Several genomic differences between SBPs and SAPs were found, although the number and function of AR genes were not different. SBPs showed larger genome size and a higher number of orthologs than SAPs. SBPs had genes associated with the utilization of L-rhamnose and D-tagatose for energy

production and nucleotide synthesis to protect them against antibiotic action. Because these sugars are also used in EPS synthesis, differences in biofilm formation-associated genes were identified. The genes for the production of EPS and cell wall proteins were different in terms of nucleotide sequences. Indeed, SAPs formed dense biofilm and survived better than SBPs in the swine intestinal environment. These results suggest that SAPs have evolved and adapted to protect themselves from new selection pressure of the swine intestinal microenvironment by forming dense biofilms, adopting a distinct resistance strategy against antibiotics. Meanwhile, it may be that the bacteria which have ability to form dense biofilm have existed before the prohibition of AGPs with a small portion, and they have become majority in the *L. salivarius* population after the AGP ban.

Study 2 mainly focused on genetic features of *L. reuteri* associated with host specificity and their antipathogenic effect. 104 *L. reuteri* strains were isolated from porcine feces, and 16 strains, composed of eight strains exhibiting the higher antipathogenic effect (HSs) and eight strains exhibiting the lower effect (LSs), were selected for genomic comparison. Draft genomes of the 16 isolates were generated and their pan-genome was investigated together with the 26 NCBI-registered genomes. *L. reuteri* pan-genome possesses 6,250 orthologs including 890 core genes and 2,081 unique genes. *L. reuteri* genomes organized six clades with MLSA, and the clade IV includes the 16 isolates. First, we identified six *L. reuteri* clade IV-specific genes including three hypothetical protein coding genes. The three annotated genes encode transposases and cell surface proteins,

indicating that these genes are the result of adaptation to the host epithelia of GIT and suggesting the possibility that these host-specific traits would be acquired by HGT.

Differences between group HS and LS were also identified in the *pdu-cbi-cob-hem* gene cluster, which is essential for reuterin and cobalamin synthesis, and six genes specific to group HS are revealed. While the strains of group HS possessed all genes of this cluster, LS strains have lost many genes of the cluster. *cbiD, cbiO, cbiP*, *sirA*, *hemB* and *hemD* were present in HSs but absent in in LSs. Based on this result, a genetic marker using four genes were designed and a method using multiplex PCR were developed for selection of antipathogenic *L. reuteri* strains.

The pan-genomic analyses of *L. salivarius* and *L. reuteri* revealed several host adaptation traits, including mutualistic properties. This finding is important to understand the evolutionary changes in reciprocal interaction in host-microbe. Furthermore, the host-specific traits of the bacteria may provide a basis for future studies of not only *L. salivarius* and *L. reuteri*, but also other symbionts. Comparison in the probiotic traits between the two groups of *L. salivarius* and *L. reuteri* showed another aspect of the mutualistic symbionts. *L. salivarius* strains isolated after the AGP prohibition increased biofilm formation. This result revealed that the ban of AGPs affected to the genetic materials of the bacteria, and contribute to select probiotic strains. The comparison of higher and lower antipathogenic ability of *L. reuteri* strains showed the genetic differences in reuterin and cobalamin production in the two groups. The studies deepen our

knowledge of probiotic bacteria and their beneficial traits and ecology of the probiotic species. Moreover, these studies provide detailed insight for the development of effective probiotics for livestock.

Key words: *Lactobacillus salivarius*, *Lactobacillus reuteri*, pan-genome, probiotics, swine, host adaptation, biofilm formation, antimicrobial activity

Student number: 2013-31036

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List of Abbreviations

3-HPA: 3-Hydroxypropionaldehyde

ABC: ATP-binding cassette

ADG: Average daily gain

AFLP: Amplified fragment length polymorphism

AGP: Antibiotic growth promoter

ANIb: Average nucleotide identity based on BLAST

APC: Antigen presenting cell

AR: Antibiotic resistance

A. pyridinolis: Arthrobacter pyridinolis

ATP: Adenosine triphosphate

B. bifidum: Bifidobacterium bifidum

B. cenocepacia: Burkholderia cenocepacia

B. coagulans: Bacillus coagulans

B. laevolacticus: Bacillus laevolacticus

B. licheniformis: Bacillus licheniformis

B. racemilacticus: Bacillus racemilacticus

B. subtilis: Bacillus subtilis

B. thermophilus: Bathymodiolus thermophilus

B. toyoi: Bacillus cereus var. *toyoi*

BHI: Brain-heart infusion

BLAST: Basic Local Alignment Search Tool

BW: Body weight

C. butyricum : Clostridium butyricum

C. difficile : Clostridium difficile

C. jejuni : Campylobacter jejuni

C. phytofermentans: Clostridium phytofermentans

CARD: Comprehensive Antibiotic Resistance Database

CDS: Coding DNA sequence

DNA: Deoxyribonucleic acid

DUF: Domain of unknown function

E. coli: Escherichia coli

E. faecalis: Enterococcus faecalis

E. faecium: Enterococcus faecium

EDTA: Ethylenediaminetetraacetic acid

EPS: Exopolysaccharide

FAO: Food and Agriculture Organization of the United Nations

FCR: Feed conversion ratio

GC: Guanine-cytosine

gDNA: Genomic DNA

GIT: Gastrointestinal tract

GRAS: Generally recognized as safe

H. pylori: Helicobacter pylori

HGT: Horizontal gene transfer

HS: Higher strain in antipathogenic activity

Hsp: Heat shock protein

IBD: Inflammatory bowel disease

Ig: Immunoglobulin

IL: Interleukin

IS: Insertion sequence

KCTC: Korean Collection for Type Cultures

KEGG: Kyoto Encyclopedia of Genes and Genomes

L. acidophilus: Lactobacillus acidophilus

L. agilis: Lactobacillus agilis

L. amylovorus: Lactobacillus amylovorus

L. brevis: Lactobacillus brevis

L. bulgaricus: Lactobacillus delbrueckii subsp. *bulgaricus*

L. casei: Lactobacillus casei

L. delbrueckii: Lactobacillus delbrueckii

L. fermentum: Lactobacillus fermentum

L. gasseri: Lactobacillus gasseri

L. helveticus: Lactobacillus helveticus

L. ingluviei: Lactobacillus ingluviei

L. jensenii: Lactobacillus jensenii

L. johnsonii: Lactobacillus johnsonii

L. monocytogenes: Listeria monocytogenes

L. paracasei: Lactobacillus paracasei

L. plantarum: Lactobacillus plantarum

L. reuteri: Lactobacillus reuteri

L. rhamnosus: Lactobacillus rhamnosus

L. sakei: Lactobacillus sakei

L. salivarius: Lactobacillus salivarius

LAB: Lactic acid bacteria

LS: Lower strain in antipathogenic activity

LTA: Lipoteichoic acid

MAMP: Microbe-associated molecular pattern

MLSA: Multi-locus sequence analysis

MRS: de Man, Rogosa and Sharpe

MucBP: Mucus-binding protein

NAD: Nicotinamide adenine dinucleotide

NCBI: National Center for Biotechnology Information

NEC: Necrotizing enterocolitis

NK: Natural killer

NOD: Nucleotide oligomerization domain

OD: Optical density

P. acidilactici: Pediococcus acidilactici

P. aeruginosa: Pseudomonas aeruginosa

P. clausseuii: Pediococcus clausseuii

P. stipitis: Pichia stipitis

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PRR: Pattern recognition receptor

RAPD: Random amplified polymorphic DNA

RAST: Rapid Annotation using the Subsystem Technology

RNA: Ribonucleic acid

rRNA: Ribosomal RNA

S. bovis: Streptococcus bovis

S. cerevisiae: Saccharomyces cerevisiae

S. enteritidis: Salmonella enterica subsp. *enteritidis*

S. faecium: Sphingobacterium faecium

S. spinosa: Saccharopolyspora spinosa

S. thermophilus: Streptococcus thermophilus

S. Typhimurium: *Salmonella enterica* subsp. *enterica* serovar Typhimurium

SAP: Strain isolated after AGP prohibition

SBP: Strain isolated before AGP prohibition

SCFA: Short chain fatty acid

SDP: Sortase-dependent protein

SRP: Serine-rich protein

TA: Teichoic acid

TE: Tris-EDTA

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TPP: Techno Plastic Products

WHO: World Health Organization

WTA: Wall teichoic acid

Introduction

Although the advantageous effects of AGPs as feed additives for livestock, AGPs have been banned globally because of the problem about bacterial AR, which occurs health problems in humans (WHO, 2013). After the legal prohibition of AGPs, farm animals are vulnerable to attack by pathogens, and livestock productivity ultimately decreased. In this aspect, a demand for an AGP alternative increases to protect animals from diseases and to maintain the productivity of livestock. A probiotic is regarded as an alternative to AGPs considering the functions of AGPs. Probiotics modulate the gut microbiota composition, and elicit the beneficial effects such as suppression of pathogenic infection, nutritional benefits, and immunomodulation. However, unproven probiotics are used in the animal industry, and therefore exact investigation of probiotic strains is needed. The important probiotic traits that most of probiotic producers are missing are host adaptation properties. Abilities for host adaptation should be considered to select probiotic strains, because these are essential for survival in the host habitats and prolong beneficial effects on hosts. Host adaptation traits include adhesion and colonization to the host habitat, nutrient utilization in the host environment, and resistance against host stress. Of note, several traits for host adaptation are associated with mutualism of the probiotic microbes and hosts.

Microbes which inhabit to the animal gut did not possess the beneficial properties to both hosts and them to begin with. All organisms have evolved to

increase their fitness in the given environment. In case of the gut symbionts, they evolve to meet the requirements as mutualistic organisms, and these requirements act as selection pressure for microbial evolution (Frank, 2007). When microbes come in the animal gut, microbial genetic content was rapidly changed under the strong selection pressure and become host-adapted symbionts. During the evolutionary process, microbes acquire and lose their genetic components, and mutation in nucleotides is occurred.

Comparative genomics provide the insight of microbial evolution and their functional properties as symbionts with the precise analysis of the genetic components. In this research, two *Lactobacillus* species were investigated. Lactobacilli are the most commonly used microorganisms as probiotics, and a part of the gut microbiota of vertebrates (Farnworth, 2008). Various health benefits ascribed to lactobacilli and members of genus *Lactobacillus* have been proposed to have potential in the reconstruction of microbiota (Farnworth, 2008; Hickson, 2011). Lactobacilli are widely used as probiotics in the livestock industry, and their abilities are proven that increasing the productivity in various animals. Especially, they are superior as porcine probiotics in all growth stages (Pluske et al., 1997; Lallès et al., 2004; Lalles, 2008).

There are many species in genus *Lactobacillus*, and among them, several species are famous as probiotics for humans and/or livestock such as *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, and *L. salivarius*. However, intensity of researches focusing on each species has

variances as shown in Table 1. In this study, two *Lactobacillus* species, *L. salivarius* and *L. reuteri*, were selected to analyze for gaining deeper understanding of the species. Although the two species are important in the animal GIT and frequently used as probiotics, the limited information for these two species is available. These two species are the most abundant microbes in the vertebrate GIT (Casas and Dobrogosz, 2000; Leser et al., 2002; Neville and O'Toole, 2010). They are known as promising probiotic species and provide mutualistic effects to the hosts. Several characters were identified, including antipathogenic ability, stimulation of the immune system, adherence to mucus, production of essential nutrients (Thornton, 1996; Dunne et al., 1999; Lin et al., 2008; Morita et al., 2008). Furthermore, they prevent several diseases such as IBD and NEC (Spinler et al., 2008; Sierra et al., 2010; Liu et al., 2013).

To understand probiotic properties of *L. salivarius* and *L. reuteri*, they were investigated in study 1 and study 2 (Figure 1). 21 *L. salivarius* strains and 16 *L. reuteri* strains were newly isolated and whole-genome-sequenced for these studies. Each study was divided to two sub-studies, composed of exploration of the species pan-genome and comparison between two groups distinguished by the probiotic characters. The former were analyzed with all NCBI-registered genomes focused on the host adaptation traits. In case of the latter, influence of the prohibition of AGPs on *L. salivarius* genomes and antipathogenic ability of *L. reuteri* were investigated. This approach to probiotic species may fulfill the gap that probiotic researches are missing, and help to selection of effective probiotic strains.

Table 1. Web-based comparison of researches on *Lactobacillus* species (in May 2017).

Species	***L. salivarius***	***L. reuteri***	***L. acidophilus***	***L. casei***	***L. plantarum***	***L. rhamnosus***	***L. fermentum***
Genomes*	44	36	18	43	193	120	25
Publication**	802	1216	3491	5962	4331	2245	1096
ATCC***	9	5	34	39	27	20	12
KCTC/KACC***	4/1	6/1	6/1	4/1	12/4	5/2	7/5

* The number of NCBI-registered genomes of each species was calculated by searching in the NCBI Genome Resources (https://www.ncbi.nlm.nih.gov/genome).

** The number of published papers of each species was calculated by searching in the PubMed (https://www.ncbi.nlm.nih.gov/pubmed).

*** The number of deposited strains of each species was calculated by searching in the ATCC, KCTC, and KACC homepage (ATCC, https://www.atcc.org; KCTC, http://kctc.kribb.re.kr; KACC, http://genebank.rda.go.kr/microbeMain.do).

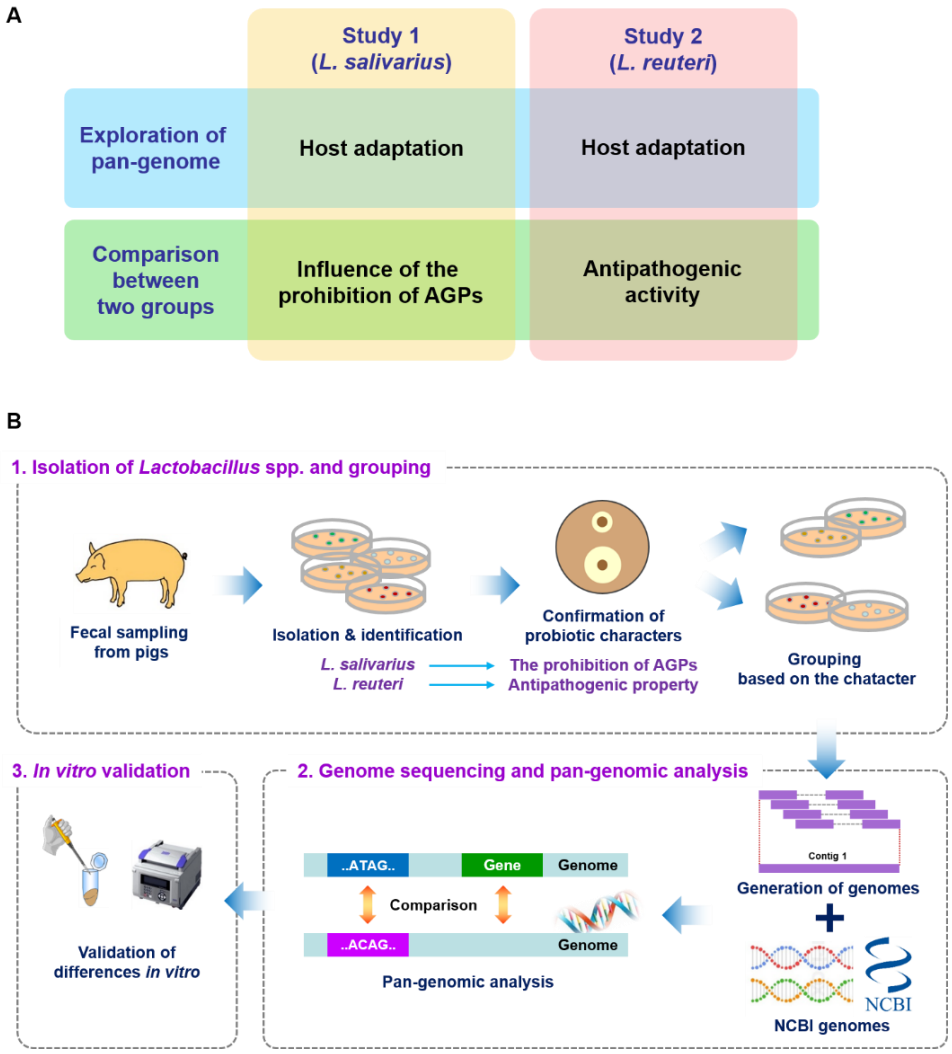


Figure 1. Overview of the research.

(A) Organization of the studies. The research consists of Study 1 and Study 2, focusing on *L. salivarius* and *L. reuteri*, respectively. Each species was analyzed in terms of the pan-genome and the comparison of its probiotic character. (B) Experimental flow of the studies. The probiotic character of *L. salivarius* and *L. reuteri* strains isolated from pig feces were tested and genome-sequenced for comparative genomic analysis.

Review of Literature

1. Probiotics and gut microbiota

1) Probiotics as alternatives of AGPs

(1) Antibiotics

Antibiotics are a type of antimicrobial drug used in the treatment and prevention of microbial infections (Walsh, 2003). Antibiotics revolutionized medicine in the past decades (Gualerzi et al., 2013). Together with vaccination, antibiotics have led to the near eradication of diseases in the developed world. They may either kill or inhibit the growth of microorganisms, especially in bacteria via various mechanisms. The mechanisms of action of antibiotics are distinguished to three categories; inhibiting cell wall and membrane synthesis and repair, blocking DNA and RNA metabolism, and inhibiting protein synthesis (Figure 2). It had been reported that antibiotics not only inhibit microbial life, but also promote growth of animals which take antibiotics with feeds and increase their productivity. People began calling the antibiotics as feed additives used for animal growth to AGP.

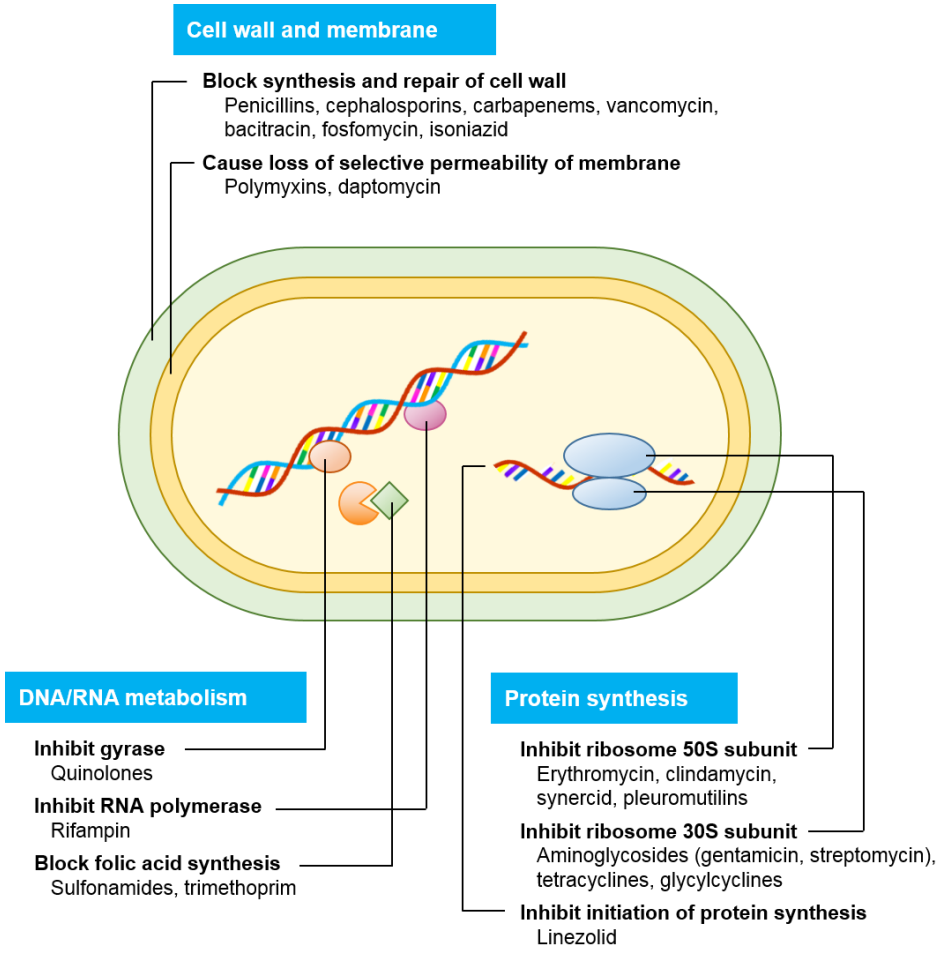


Figure 2. Antibiotic mechanisms of action.

Antibiotics cause critical damages to microbes with various mechanisms of action. These mechanisms are distinguished to three categories; inhibiting cell wall and membrane synthesis and repair, blocking DNA and RNA metabolism, and inhibiting protein synthesis.

(2) A need of AGP alternatives

AGPs have been added to livestock diets since the 1950s (Dibner and Richards, 2005). They have provided increased productivity to farmers, and the agricultural industries and the consumers have benefited from them. Antibiotics have indirect effects through the microbiota and direct effects on the host. However, the functional and interactive effects of AGPs on the host and the microbiota are less clear. A variety of environment factors, including diet, stress, and activity are known to influence the composition of the microbiota and the host, and there is potential for these factors to influence the efficacy of AGPs (Brown et al., 2017).

In the last few decades, scientists and politicians have expressed concerns about the use of antibiotics in animal feed. These include the potential development and transfer of AR of bacteria, which could become resilient to medicines used to treat humans, leading to increased illnesses and mortality in humans (WHO, 2013). An action supporting the ban of AGPs from livestock feed was started in Europe. Sweden was the first country to ban the use of all AGPs, and since then many countries have prohibited the addition of AGPs to animal feeds (Dixon, 2000). The ban of AGPs causes several other problems including vulnerable status of farm animals to attack by pathogens and decreased livestock productivity. It took 10 years that recovery of the productivity in Sweden (Inborr, 2000). Furthermore, the cost for pork production in Denmark ultimately increased (Kjeldsen and Callesen, 2006). In this regard, an AGP alternative is needed to protect animals from diseases following the governmental ban of AGPs.

(3) Definition of probiotics

Probiotics are regarded as an alternative to AGPs considering the functions of AGPs, which are health benefits to the animals and the ability for modulation of the intestinal microbiota. FAO/WHO defined the probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). Probiotic have received attention in the last few decades because it is one of the effective functional foods for humans and feed additives for livestock (Tomayko et al., 2013). Various microbes are considered as probiotics, such as LAB, bacilli, and yeast.

Major type of probiotics is LAB, which are Gram-positive bacteria consisting of several genera, *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Carnobacterium*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Von Wright and Axelsson, 2011). These bacteria are GRAS, and produce lactic acid through lactic acid fermentation. They are generally found in the environment such as fermented foods, soil, animal digestive tracts (Parente et al., 2001; Heilig et al., 2002; Callon et al., 2004). Among them, the most commonly used strains are involved in genus *Lactobacillus* (Farnworth, 2008).

2) Probiotics and gut microbiota

(1) Gut microbiota

Microbiota in the vertebrate GIT are distinguished from communities of free-living bacteria from natural environment, such as soil and seawater (Ley et al., 2008a). Human Microbiome Project had reported that 29% of the sequenced bacterial species inhabited in the GIT of human (Peterson et al., 2009). The GIT is a habitat for a diverse and complex community of microbes. The intestinal microbiota is composed of mainly bacteria, but also viruses, archaea, fungi and protozoa have their portion and roles. The gut of adult humans has 10^{13} microbes including 500 ~ 1,000 bacterial species (Xu and Gordon, 2003; Eckburg et al., 2005). These numerous microbial species possesses more than 20 million unique genes, termed the microbiome, which is estimated to exceed the total number of human genes over 100 times (Proctor, 2011). Their genes provide to animals many functions that animals do not acquire during the evolutionary process.

The distribution of the microbiota is not homogenous in the GIT, due to bacterial density and diversity increase from the proximal to distal gut (Figure 3). In vertebrates, the highest cell populations (10^{10} to 10^{12} cells/ml) are found in the distal regions (e.g., colon or cecum), making it one of the densest microbial ecosystems on earth (Whitman et al., 1998). The gut ecosystem is highly dynamic because numerous factors influence the microbiota composition, including host phylogeny, sex, age, gut health, diet and other factors (Ley et al., 2008b; Turnbaugh et al., 2008; Benson et al., 2010; Ochman et al., 2010).

Gut microbes have a symbiotic relationship with their hosts. The term ‘symbiosis’ refers to an intimate and long-term relationship between various microorganisms, regardless of the effects of the interaction on the fitness of the partners. Symbiosis is defined to include relationships that are 1) mutualistic: beneficial to both, 2) commensal: one benefits and another is unaffected, and 3) parasitic: one benefits and another is harmed (Hill and Artis, 2009; Walter et al., 2011). It is notable that although net effect of the gut microbiota is beneficial, specific components of the microbiota may exist between mutualism and parasitism (Walter et al., 2011).

The host provides an attractive environment for the microbes, rich in nutrients and stable in temperature. In return, the microbiota contribute to host health. Studies on the composition of the intestinal microbiota affecting health conditions suggest that gut microbiota is a major determinant in health and disease by degradation of indigestible carbohydrates, supplying essential nutrients, promoting angiogenesis, protecting against pathogens, regulation of the immune system, and the organ development (Hooper and Gordon, 2001; Schrezenmeir and de Vrese, 2001; Macpherson and Harris, 2004; Sekirov and Finlay, 2009). As a consequence, there is growing interest in altering the composition of intestinal microbiota as a therapeutic strategy. For example, the fecal transplantation to patients with *C. difficile* infection leads to maintain health through microbiota modification (Bakken et al., 2011).

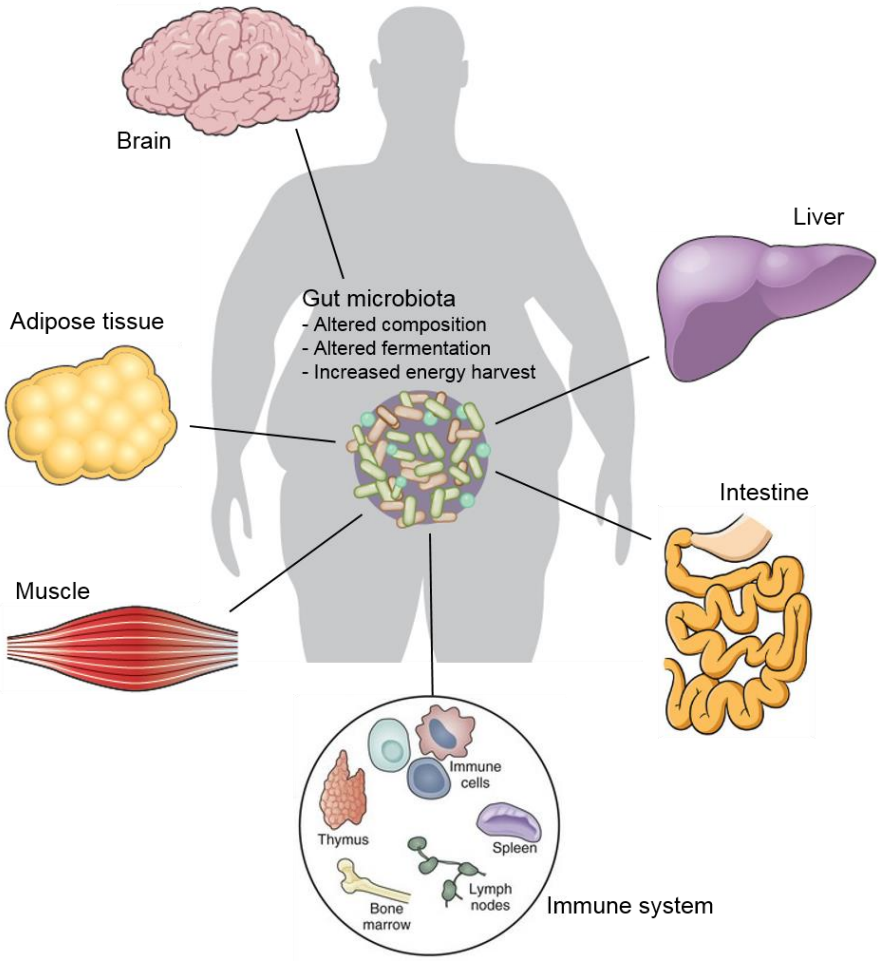


Figure 3. Organs affected by gut microbiota.

Alterations to the composition and metabolic capacity of gut microbiota influence several processes in various organs. The figure is modified from Tremaroli and Bäckhed (2012).

(2) Probiotics and gut microbiota

Well-balanced relationship between gut microbiota and host is necessary for maintaining animal health. The disruption of this balance can result in disease, implying that alteration of gut microbiota can be utilized to prevent or cure unhealthy conditions of animals. Probiotics are one of the strategies that could be used to alter the gut microbiota and restore the 'good' microbial community. A mixture of lactobacilli and bifidobacteria have been shown to prevent *C. difficile* infection in a mouse model (Kondepudi et al., 2014). Modulating gut microbiota is also considered an therapy in IBD patients (Hegazy and El-Bedewy, 2010).

Furthermore, several studies showed that probiotics are effective to replace AGPs for livestock. Treatment of probiotics including genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* to broilers stabilized ruminal pH, increased the absorption of some nutrients and displayed a growth-promoting effect that was comparable to avilamycin treatment (Ayed et al., 2004; Mountzouris et al., 2007). In pigs, two probiotic mixtures of *S. faecium* and *L. casei* or *S. cerevisiae* and *Bacillus* spp. showed similar effect on growth performance and nutrient digestibility compared with antibiotics-treated group (Xuan et al., 2001). *B. licheniformis* and *B. subtilis* improved BW, ADG, feed efficiency of pigs, and prevented diarrhea as well as an AGP (Link et al., 2005).

(3) Co-evolution of gut symbionts and hosts

Symbiosis is maintained by transmission of environmental microbes. Transmission is mainly occurred with mixed mode involving both vertical and horizontal transfers (Bright and Bulgheresi, 2010). Vertical transmission means a situation that symbionts are transferred from parent to offspring directly. Meanwhile, horizontally transmitted symbionts are acquired from the free-living populations. Symbionts that are vertically transmitted have evidences of co-evolution although horizontally transmitted symbioses do not. Co-evolution of the microbes and hosts regulates their mutual evolution (Baumann, 2005; Dale and Moran, 2006; Moran et al., 2008).

An evidence that gut microbes co-evolved with their hosts has mainly been inferred by analysis of whole bacterial communities. Different host species sustain host-specific composition of microbes (Dethlefsen et al., 2007; Ley et al., 2008b; Ley et al., 2008a). These communities are various between hosts, because of restricted migration of microorganisms between hosts and strong ecological interactions of microbes within hosts, as well as host variability in terms of diet, genotype and colonization history (Dethlefsen et al., 2007). The impact of host species on microbial community is most apparent when identical species hosts living in apart regions clustered together (Ley et al., 2008a). Patterns of community similarity provided evidence that gut bacterial communities have co-diversified with their hosts (Ley et al., 2008b; Ochman et al., 2010). This suggests that host-specific evolutionary interactions exist between animals and their

microbiomes.

Some members of the gut microbiota are generalists that show promiscuous lifestyles (Ley et al., 2008a). Such an evolutionary strategy was adopted by commensal *E. coli* that have evolved to occupy niches within a broad host range. Although the primary habitat of *E. coli* is in the vertebrate gut, it can also exist in the outer environment (Touchon et al., 2009; Tenaillon et al., 2010). Whereas some bacteria within the digestive tract, such as *H. pylori* have co-evolved with their hosts for long evolutionary period (Falush et al., 2003), and most of mutualistic bacteria are also under the same situation. In case of this, they try to adapt to the host environment and provide the benefits to hosts because this evolutionary direction is 'good' for both hosts and them (Figure 4). The genome of evolving bacterium is changed under strong selection pressure, which elicits gene loss and/or acquisition through HGT, and mutation in nucleotides, etc. Finally, the genome is specialized to the hosts, termed 'host specialization'. This yielded the smallest (Nakabachi et al., 2006; McCutcheon et al., 2009), most stable (Tamas et al., 2002; Moran et al., 2009), most highly repeated (Cho et al., 2007) and most highly recombined genomes (Klasson et al., 2009) of commensal bacteria.

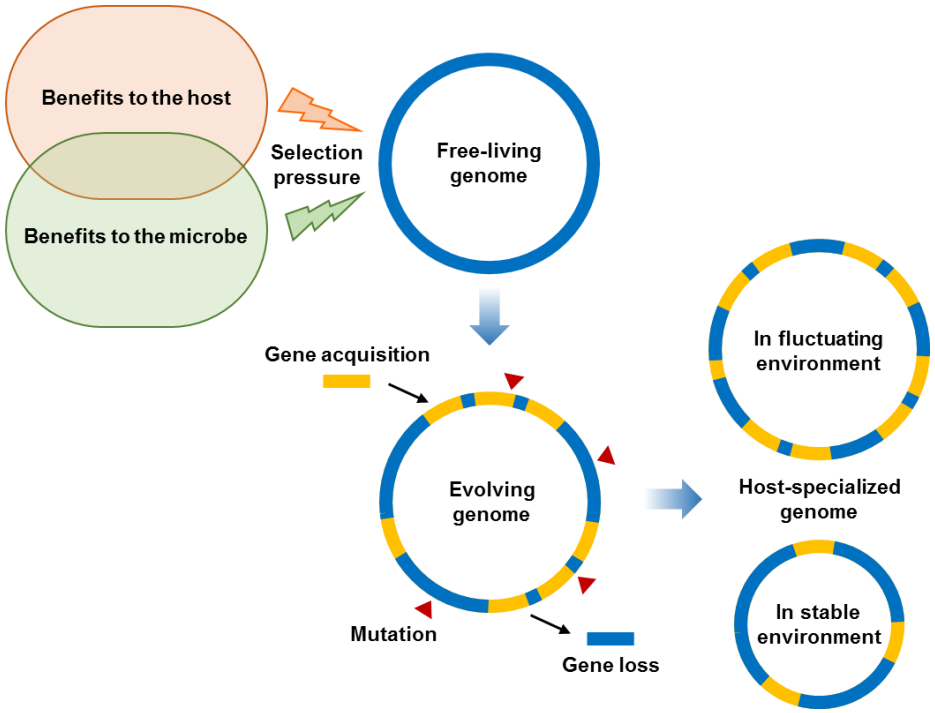


Figure 4. Changes in genetic components of symbionts during evolutionary process.

When a free-living microbe came into the animal intestine, a genome of the microbe becomes to be under the selection pressure for increase microbial fitness, and the genome is changed through gene acquisition and loss via HGT, and point mutation. Finally, the genome is adapted to the intestinal environment, termed a host-specialized genome. In fluctuating environment, the genome acquires more genes and the genome size becomes larger, and in stable environment, the genome becomes smaller with less genetic changes.

2. Functions and roles of probiotics for animals

1) Beneficial effect on hosts

The most important features of probiotics are the ability to confer a health benefit on a host. To examine the beneficial health effects of probiotics, a variety of strains have been applied to diseases and unhealthy conditions. There are various mechanisms of action of probiotic microbes, including suppression of pathogenic infection, nutritional benefits, and immunomodulation (Figure 5).

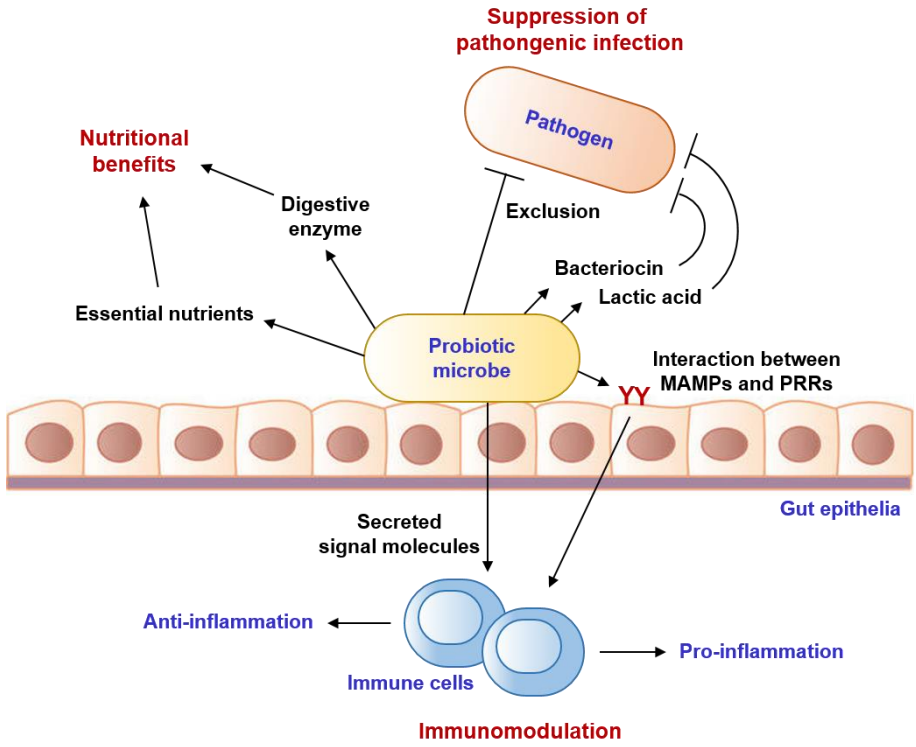


Figure 5. Mode of action of probiotic microbe.

Beneficial effects of probiotics are distinguished to three categories; 1) Suppression of pathogenic infection, 2) Nutritional benefits to hosts, 3) Modulation of host immune system.

(1) Suppression of pathogenic infection

The indigenous intestinal bacteria inhibit pathogens by competition to colonization and nutritional source and production of toxic or stimulation of the immune system which suppresses pathogenic infection (Parvez et al., 2006). Probiotics have been shown to be involved in protection against a variety of pathogens in chicken including *E. coli*, *Salmonella*, *Campylobacter*, *Listeria*, *Clostridium* and *Eimeria* (Chateau et al., 1993; Stern et al., 2001; Dalloul and Lillehoj, 2005; Casey et al., 2007). *L. rhamnosus* GG was shown to be effective in preventing antibiotic-associated diarrhea (Hawrelak et al., 2005). Several LAB strains can reduce the level of *E. coli* O157 in cattle and calves (Brashears et al., 2003), and decreased diarrhea in *E. coli* O157:H7-infected rabbits (Ogawa et al., 2001). The growth of *E. coli* O157:H7 and the production of toxins were inhibited by co-incubation with *C. butyricum* (Takahashi et al., 2004). The mixture of *L. rhamnosus* R0011 and *L. acidophilus* R0052 reduced gastric inflammation and bacterial colonization in *H. pylori*-infected animals (Johnson-Henry et al., 2005). *E. faecium* and *S. cerevisiae* induced an inflammatory response in steers (Emmanuel et al., 2007). The *P. acidilactici* protects against illness associated with coccidiosis (Lee et al., 2007). *L. acidophilus* DSM13241 altered the balance of gastrointestinal microflora in cats (Marshall-Jones et al., 2006). Probiotic also can exhibit antibacterial activity against fish pathogenic bacteria (Sugita et al., 2002).

(2) Immunomodulation

The intake of probiotics enhances host immune defense or modulates host immune system, such as improving resistance against diseases and reducing inflammation (Jukna et al., 2005). Some probiotic strains ameliorate inflammation-related diseases (Kruis et al., 2004). Probiotics prevent or reduce severity of infectious and antibiotic-associated diarrhea and respiratory tract infections (Jonkers, 2016). Probiotics also have the potential to control allergic inflammatory responses caused by disorders associated with disruption of microbiota (Isolauri et al., 2000; Sanders et al., 2013).

L. rhamnosus GG was shown to prevent atopic eczema among children at high risk for the disease (Kalliomäki et al., 2007). Probiotic mixture containing *L. acidophilus*, *L. casei*, *E. faecium*, and *B. thermophilus* increased intestinal IgA secretion both in sows and piglets and elevated IgG and IgM levels in turkey (Cetin et al., 2005). *E. faecium* NCIMB10415 and *B. toyoi* NCIMB40112 improved secretion of intestinal IgA which is related to a successful mucosal defense in weaning piglets (Scharek et al., 2007). Probiotics also influence transport properties of small intestine epithelium and increase absorption of glucose (Lodemann et al., 2006; Lodemann et al., 2008). The probiotics that increase Ig levels have more positive effect on growth performance, production and resistance against diseases (Cetin et al., 2005). Severity of *E. coli* O157:H7 infection in mice was reduced by *feeding L. rhamnosus* HN001, which enhanced humoral and cellular immune responses (Shu and Gill, 2002). *B. coagulans* shows

immunoregulatory abilities on cell-mediated immunity and humoral immune response in poultry (Panda et al., 2008). *C. butyricum* mediates the humoral immune responses and improve the growth performance in croakers (Song et al., 2006).

(3) Nutrition and metabolism

Microorganisms in the digestive tract affect to the availability and digestibility of various dietary nutrients. Probiotics has a positive effect on various digestive processes, especially cellulolysis and synthesis of microbial proteins (Yoon and Stern, 1995). LAB are known to release various enzymes into the intestinal lumen that exert synergistic effects on digestion, alleviating intestinal malabsorption. Bacterial enzymatic hydrolysis enhances the bioavailability of protein and fat and increase the production of free ammo acids (Friend and Shahani, 1984). LAB stabilize ruminal pH with lactic acid production, which increases the absorption of some nutrients (Mountzouris et al., 2007).

SCFAs are also produced by several probiotic strains. When absorbed these SCFAs contribute to the available energy of the host (Rombeau et al., 1990) and may protect against pathological changes in the intestinal mucus layer (Leavitt et al., 1978). In addition, SCFA helps to maintain an appropriate pH in the intestinal lumen, which is critical in the expression of many bacterial enzymes and in metabolism in the GIT (Mallett et al., 1989).

Several studies showed that probiotics are associated with metabolic diseases, and facilitate the prevention and control of hyperglycemia (Ruan et al., 2015), improve levels of high-density lipoprotein and TNF-α in patients of fatty liver disease (Gao et al., 2016), and reduce glucose and insulin in diabetes patients (Sun and Buys, 2016).

2) Host adaptation

Microbes in GIT and hosts co-evolve to increase their fitness with reciprocal interactions. As described above, the host provides an attractive environment for the microorganisms, and in return, the microbiota contributes to host health. Inversely, microbe that is suitable to inhabit to the host habitat survive in the environment, and hosts adopt selectively the microbes which is beneficial to their health. This mutualistic interaction leads to host adaptation of the gut microbes. For example, some bacteria produce and secrete the essential nutrient, vitamin B_{12} (Albert et al., 1980; Martens et al., 2002). Bile salt hydrolase produced by several lactobacilli provides bile detoxification, gastrointestinal persistence and membrane alterations for the microbes, and for hosts it alters digestive functions and lowers cholesterol (Bateup et al., 1995; Smet et al., 1995; Tanaka et al., 1999; Begley et al., 2006).

Each host species has original intestinal environment, and microorganisms have tried to adapt to the host environment (Toft and Andersson, 2010). The microbiome of the commensal bacteria in GIT is affected by the animal's diet, age, immune function, and genetics (Benson et al., 2010; Biagi et al., 2010; Kim et al., 2011; Belanche et al., 2012; Jeraldo et al., 2012; Maynard et al., 2012; Haenen et al., 2013), as well as complex interactions among microbial species (Yeoman et al., 2011; Tymensen et al., 2012). These complex features change interspecies and inter-individual microbiome characteristics (Yildirim et al., 2010; Jami et al., 2013). Microbial traits for host adaptation can be divided into three categories;

abilities for adhesion and colonization to host habitats, utilization of nutrients in host environments, and resistance against host stress (Figure 6).

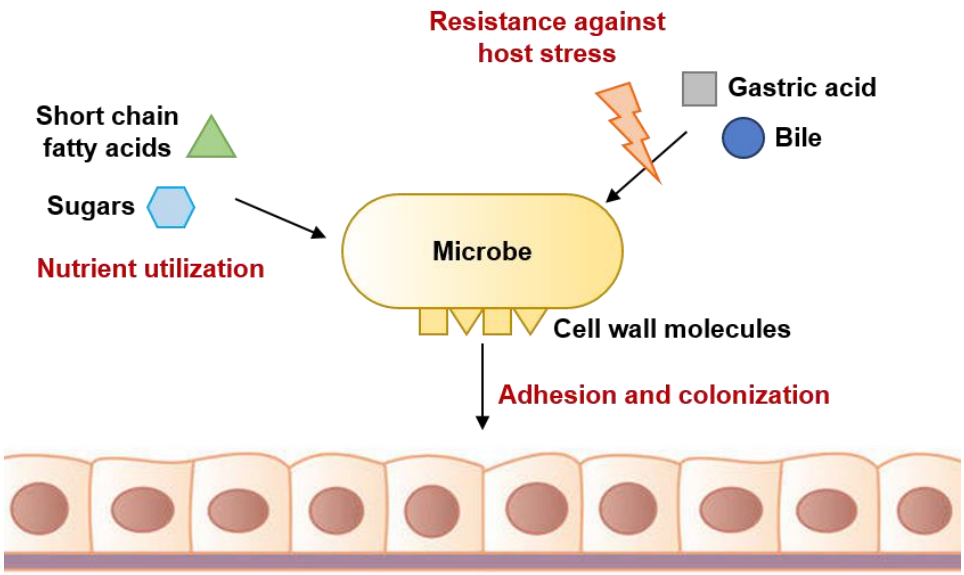


Figure 6. Bacterial traits for host adaptation.

Host adaptation is categorized by three mechanisms; 1) Adhesion and colonization to host habitats, 2) Utilization of nutrients originated from hosts, 3) Resistance against host stresses.

(1) Nutrient utilization

Microbiome in the GIT is influenced by host nutrition. Lactobacilli have evolved to be fastidious organisms that require complex nutritional requirements such as easily fermentable sugars, amino acids, peptides, nucleic acid derivatives, vitamins, salts and fatty acid esters for growth (Holzapfel and Wood, 2012), indicating that these indigenous commensal bacteria have adapted to host environment which is rich in nutrients.

Of the various nutrients, starches showed one of the most significant modulatory effects on the GIT microbiome (Haenen et al., 2013). Starches containing high amylose contents promote microbes and microbial metabolites commonly associated with improved animal health (Martínez et al., 2010; Regmi et al., 2011; Young et al., 2012). Simple carbohydrate sources also increase GIT fermentation. These carbohydrates lead to the production of SCFAs, which is necessary to enable the microbiome to produce energy (Flint and Bayer, 2008). The increasing acidity caused by SCFAs reduces the fermentative capabilities of fibrolytic microbes, while favoring more acid-tolerant microbes such as *S. bovis* and lactobacilli (Herrera et al., 2009).

(2) Adhesion to host habitats

Among probiotic bacteria, the adherence ability to host habitats and the specificity had been well documented in lactobacilli. The gastric regions of rodents, pigs, birds, horses, and other animals are lined with a non-glandular, squamous stratified epithelium (Tannock, 1992). Lactobacilli have adapted to densely colonize to these regions and form layers of biofilms (Yuki et al., 2000; Hilmi et al., 2007). The bacteria adhere directly to the stratified squamous epithelial lining at the proximal regions of the GIT, such as forestomach of rodents, crops of chickens, and pars oesophagea of pigs (Wesney and Tannock, 1979; Lin and Savage, 1984; Tannock, 1992). Conversely, the human stomach is lined with a glandular mucosa, and epithelial cell layers rich in lactobacilli have not been described humans (Walter, 2008). The ability of lactobacilli to adhere to epithelial cells in the proximal GIT is host-specific (Suegara et al., 1975; Wesney and Tannock, 1979; Lin and Savage, 1984). For example, isolates originated from rodents adhere to epithelial cells of mice and rats, but they do not adhere to crop epithelial cells of poultry. Otherwise, poultry isolates do not adhere to epithelial cells of the rodents or pigs (Tannock, 1997).

(3) Resistance against host stress

One of the reasons of why *Lactobacillus* is a popular probiotic genus is based on their resistance ability against host defense mechanisms, such as gastric acid and bile. This trait is desirable for probiotic strains, because it is important to survive in the animal GIT. Several lactobacilli produce choloylglycine hydrolase, as known as bile salt hydrolase (Bateup et al., 1995; Smet et al., 1995; Tanaka et al., 1999). This mutualistic enzyme provides bile detoxification, gastrointestinal persistence and membrane alterations for the microbes, and alters digestive functions and lowers cholesterol for hosts (Begley et al., 2006). *B. bifidum*, *L. acidophilus*, *L. delbrueckii* LB9 and *S. thermophilus* ST20 showed the tolerance against gastric and intestinal conditions including gastric acid, bicarbonate, bile and pancreatic juice (Marteau et al., 1997). *B. laevolacticus* DSM6475 and several *Sporolactobacillus* strains were resistant to acid condition, and *B. racemilacticus* and *B. coagulans* strains were tolerant to bile (Hyronimus et al., 2000). Besides, some lactobacilli, bifidobacteria and enterococci have the ability for reducing damages from gastric acid and bile (Flahaut et al., 1996; Jin et al., 1998; Chung et al., 1999). Furthermore, bile treatment to some bacterial species increases expression of genes that are responsible for adaptation to abnormal conditions via DNA repair, synthesis of cell envelope, and detoxification (Merritt and Donaldson, 2009), implying that this harsh environment driven by the host secretion acts as evolutionary pressure for survival in the animal GIT.

3. Lactobacilli as probiotics

1) Probiotic factors of lactobacilli

Lactobacillus is the most commonly used genus as a probiotic, and part of commensal microbiota (Farnworth, 2008). Various health benefits ascribed to lactobacilli and members of this genus have been proposed to have potential in the reconstruction of the gut microbiota (Farnworth, 2008; Hickson, 2011). Probiotic lactobacilli, like other Gram-positive bacteria, possess MAMPs that interact with PRRs such as TLRs, NOD-like receptors, and C-type lectin receptors. Host cells are affected by MAMPs and produce small molecules that elicit an immune response. Several factors in lactobacilli have been shown to impact the various response *in vitro* and *in vivo*, through their cell surface components and secreted molecules (Lebeer et al., 2008).

(1) Secreted molecules

Environmentally released small peptides or metabolites are emerging as key components of probiotic traits (Figure 7). Probiotics generate a physiologically restricted environment to other bacteria by decreasing pH and changing the gut's redox potential through secretion of the small molecules or metabolites (Lebeer et al., 2008). In the gut, this may be one of the possible mechanisms of modifying the microbiota via immune signaling.

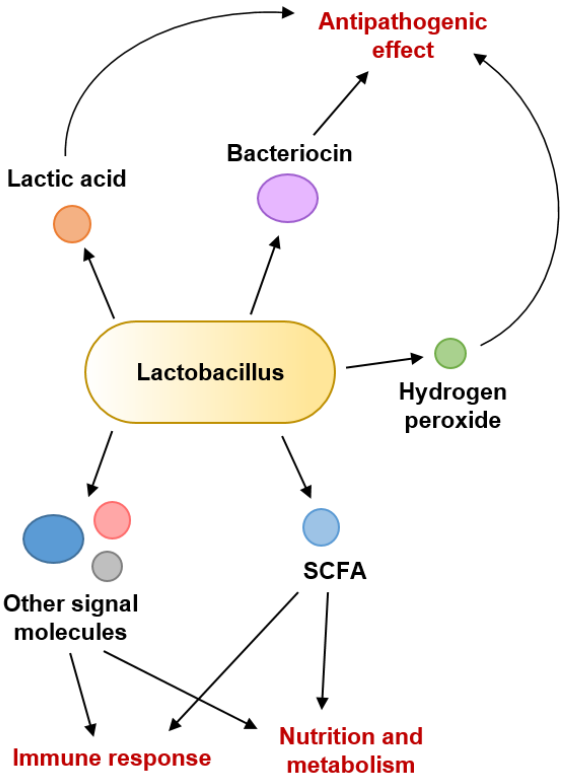


Figure 7. Molecules secreted from *Lactobacillus* and their major effects.

Various molecules are secreted from lactobacilli, and these secreted molecules function to the host adaptation and provide the health benefit to a host.

Lactic acid Lactobacilli produce lactic acid that causes a reduction in the intestinal pH and inhibits acid-sensitive microorganisms. This has been demonstrated in a study of the effectiveness of many *Lactobacillus* strains against *S.* Typhimurium (Fayol-Messaoudi et al., 2005). Lactobacilli have been found to inhibit *H. pylori* growth both *in vitro* and *in vivo*, and the amount of lactic acid produced correlated with the intensity of their inhibition (Aiba et al., 1998; Sgouras et al., 2004).

SCFA Lactobacilli lead to the production of SCFAs, such as acetate, propionate and butyrate, by modifying the microbial composition (Ceapa et al., 2013). Butyrate is an energy source for epithelial cells in the colon and has been found to induce expression of genes encoding β-defensin and leukocyte antimicrobial peptides in pigs, as well as to improve insulin sensitivity in humans (Vrieze et al., 2012; Zeng et al., 2013).

Bacteriocin Lactobacilli also modify the composition of intestinal microbiota by producing antimicrobial compounds called bacteriocins, which are typically effective against closely related bacteria. However, some bacteriocins are active against wide range of microorganisms including Gram-negative bacteria, molds, and yeasts (Lebeer et al., 2008; O'Shea et al., 2012). Various bacteriocins produced by different *Lactobacillus* species have been described

(Klaenhammer, 1988). For example, *L. salivarius* UCC118 produces a peptide that inhibits a wide range of bacteria, including *Bacillus*, *Enterococcus*, *Listeria*, *Salmonella*, and *Staphylococcus* species (Garneau et al., 2002).

Other molecules Lactobacilli can produce hydrogen peroxide and create a nonspecific barrier as an antimicrobial activity (Pridmore et al., 2008). p40 and p75, two secreted proteins of *L. rhamnosus* GG and some *L. casei* strains, promote intestinal epithelium cell homeostasis by inhibiting epithelial cell apoptosis induced by pro-inflammatory cytokines in human and mouse colon epithelial cells (Yan et al., 2007; Bäuerl et al., 2010). A study discovered that the soluble protein p40 upregulates mucin production in goblet cells (Wang et al., 2014b). Human-derived *L. reuteri* strains that convert dietary histidine into histamine and suppresses the host inflammation. *L. reuteri* 6475 has been shown to regulate TLR-2-induced TNF-α secretion by producing histamine (Thomas and Versalovic, 2010). In another study, soluble molecules from *L. casei* blocked rotavirus infection in a species-specific manner. A possible mechanism of action involves small molecules produced by these bacteria modifying glycans on the intestinal cell surface and preventing the attachment of rotavirus to the host cell's surface (Varyukhina et al., 2012).

(2) Cell surface components

The outer surface of bacteria represents the first interface between hosts and microbes in the gut. Typical cell surface structures of lactobacilli, like other Gram-positive bacteria, contain a thick and multilayered peptidoglycan layer decorated with surface proteins, TAs, and polysaccharides (Figure 8). *Lactobacillus* has great diversity in composition of cell surface molecules, and therefore the chemical structure of their cell surfaces results in species and strain specificity of probiotics (Lebeer et al., 2008).

Several MAMPs on the surface have been identified in lactobacilli as effector molecules of probiotic action. MAMPs are recognized by PRRs, which trigger an innate immune response. Targets of PRRs are usually cell surface components, such as peptidoglycan, TA, lipids, and proteins. Cell surface structures play a key role in the host-microbe interaction, the adaptation of probiotics to the host, and immunomodulation by probiotics (Lebeer et al., 2010; Sengupta et al., 2013). Understanding the variation of MAMPs located on their cell surfaces would be a significant contribution to understanding probiotic modes of action and the strain specific health effects of these organisms.

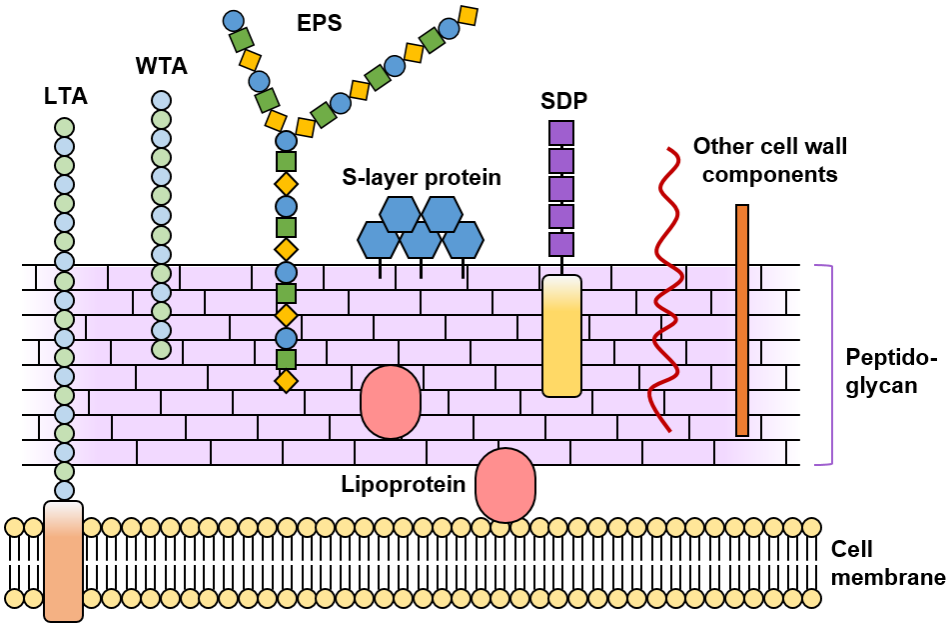


Figure 8. Cell wall structure and its components of lactobacilli.

Cell wall components of lactobacilli consist of various sugars, proteins, lipids, etc. in common with other Gram-positive bacteria. Peptidoglycan and TA (including LTA and WTA) are the most abundant components in the cell wall.

Peptidoglycan Peptidoglycan is composed of glycan chains of repeating pentapeptide-linked N-acetylglucosamine and N-acetylmuramic disaccharide units that determine the shape and the integrity of bacteria (Kleerebezem et al., 2010). It has been shown to modulate immune responses. Anti-inflammatory effects of peptidoglycan from *L. salivarius* Ls33 have been demonstrated in a mouse (Fernandez et al., 2011). Post-modifications in the short peptides associated with the glycans of *L. plantarum* is a strategy to avoid a pro-inflammatory effect in some microorganisms and provide strain specificity in the immunomodulatory action of probiotics (Asong et al., 2009). After host cell internalization of peptidoglycan fragments, peptidoglycan is recognized by NOD-like receptors (Regulski et al., 2012).

Teichoic acid After peptidoglycan, the second major component of Gram-positive bacterial cell walls is TA (Lebeer et al., 2008). TAs are made of polyglycerol phosphate or polyribitol phosphate repeating units. There are two forms of TAs, WTA and LTA; These are distinguished by which the repeating units are covalently linked to the cell wall or anchored to the cytoplasmic membrane, respectively (Lebeer et al., 2010; Brown et al., 2013). These TAs are MAMPs and induce innate immune responses. Even though all lactobacilli have TAs, they differ in their ability to induce TLR signaling, which may be related to variation in their chemical structure (Wells, 2011). The highly diverse and strain specific structures of TAs lead to distinct inflammatory effect, such as pro- or anti-

inflammation (Lazarevic et al., 2002; Remus et al., 2011; Brown et al., 2013).

LTA is considered more immunostimulatory than WTA, because LTA structure has a lipid component (Lebeer et al., 2012). Lipid chains of LTA interact with the TLR2-binding pocket and induce an inflammatory response (Sengupta et al., 2013). WTA plays a primary role in the maintenance of cell morphogenesis (Bron et al., 2012b). WTA and LTA coexist in most Gram-positive bacteria, however, some lactobacilli species contain only LTAs, such as *L. rhamnosus* and *L. casei* (Kelemen and Baddiley, 1961; Bron et al., 2012a).

Exopolysaccharide In some cases, the cell wall of lactobacilli is decorated with capsular polysaccharides, termed EPS, and its complex structure contributes to diversity in the lactobacilli cell wall (Lebeer et al., 2008). Synthesis of EPSs is related with genes encoding glycosyltransferases and proteins that are responsible for export and regulation. These genes overlap with the genes involved in the biosynthesis of other cell wall polymers, such as peptidoglycan or WTAs (Chapot-Chartier and Kulakauskas, 2014). EPSs have been found to be involved in other important functions like tissue adherence and biofilm formation (Lebeer et al., 2008; Yasuda et al., 2008). EPSs could act as ligands for C-type lectin receptors of the immune system (Sengupta et al., 2013). EPSs of *L. casei* Shirota have been shown to mediate the suppression of pro-inflammatory responses in macrophages (Yasuda et al., 2008).

S-layer protein S-layer proteins are the most abundant surface proteins in some of *Lactobacillus* species, such as *L. brevis*, *L. acidophilus*; however, they are not present in *L. casei*. They are commonly involved in bacterial adherence to host cells (Hynönen and Palva, 2013). Additionally, S-layer proteins are shown to have an immunostimulatory function. An S-layer protein produced in *L. acidophilus* has been identified as the ligand for a C-type lectin receptor and found to be involved in immune functions of dendritic cells (Konstantinov et al., 2008).

Sortase-dependent protein SDPs are one of the best characterized surface proteins in lactobacilli. SDPs contain a LPXTG cell wall-sorting signal at the C-terminus that is recognized by a membrane associated sortase SrtA. This enzyme cleaves the protein between threonine and glycine and anchors the threonine residue to the peptidoglycan (Navarre and Schneewind, 1994). A growing number of studies reported that LPXTG-anchored cell wall proteins act as MucBPs and adherence to the host intestinal cells (van Pijkeren et al., 2006; Kleerebezem et al., 2010; Call et al., 2015). SDPs also have been shown to impact cytokine production in murine dendritic cells (Call et al., 2015). Gram-positive bacteria utilize sortase-mediated mechanisms to assemble pili on the cell surface. A study with *L. rhamnosus* GG revealed that pili play a key role in this strain's capacity to adhere to macrophages (Reunanen et al., 2012). In addition, they mediate the anti-inflammatory effect of *L. rhamnosus* GG by inducing the expression of IL-10 and decreasing the expression of IL-6 (García et al., 2015).

Lipoprotein In some Gram-positive bacteria, lipoproteins are dominantly immunological compound via TLR2 (Hashimoto et al., 2006). While their role in immune interactions has not been thoroughly studied in lactobacilli, it was predicted that lipoproteins from *L. johnsonii* NCC 533 were adhesion factors (Pridmore et al., 2004).

2) Lactobacilli as probiotics for livestock

In animal nutrition, microorganisms used as probiotics were linked with a proven efficacy on the gut microflora. They not only prevent diseases, but also increase growth performance and quantity and quality of the products, such as milk, meats, and eggs. There are numerous studies on probiotics accelerating growth of farm animals. Administration of probiotic strains significantly improves feed intake, FCR, ADG and BW in chicken, pig, cow, sheep, goat and equine (Chiofalo et al., 2004; Casey et al., 2007; Samli et al., 2007; Torres-Rodriguez et al., 2007). Furthermore, probiotics have a beneficial effect on milk yields, fat and protein content (Sara et al., 2002; Kritas et al., 2006). Supplementation of probiotics also improves egg production and egg quality (Haddadin et al., 1996; Kurtoglu et al., 2004), and decrease egg contaminations from pathogens (Van Immerseel et al., 2006). Probiotics are also used to meet the needs of safe and qualitative meat. Probiotics increased the quantity of carcass and water holding capacity and decreased cooking loss and meat hardness (Jukna et al., 2005), and reduced morbidity and mortality of growing rabbits during the fattening period (Matusevičius et al., 2006).

In the pig industry, lactobacilli have been used as feed additives for a long time. *Lactobacillus* probiotics used in all stages of pig growth (Table 2). Especially in the weaning period, the piglets have to deal with abrupt separation from their mother (sow) in a new environment, and switch their feed from highly digestible feed (e.g. milk) to a solid and complex feed. This environmental change in terms

of nutrition, psychology, microbiology, and immunology causes severe stress to the piglets, with many adverse effects including reduced feed intake, impaired intestinal health, diarrhea and BW loss (Pluske et al., 1997; Lallès et al., 2004; Lalles, 2008). It also leads to villous atrophy in the small intestine, thus impairing digestion and absorption of the nutrients in the gut (Montagne et al., 2007). The intestinal health can be ameliorated by use of probiotics, which stimulate the development of a healthy microbiota by beneficial bacteria as described it above, implying the reason why probiotics were intensively used in a certain period. Of note, not only in weaning period, several studies showed the beneficial effects of *Lactobacillus* probiotics in other growth stages of pigs (Table 2).

Table 2. Studies of *Lactobacillus* strains used as porcine probiotics

Stage of pigs	Probiotic strain	Effect(s)*	Reference
Nursery	*L. acidophilus* LAC-300	BW ↑ and FCR ↓, diarrhea ↓	(Abe et al., 1995)
	L. casei	*E. coli* prevalence ↓	(Bomba et al., 1999)
	L. reuteri I5007	ADG ↑, Clostridium infection ↓, IL-1b in the ileum ↓, diarrhea ↓	(Liu et al., 2014)
	L. jensenii TL2937	Growth performance ↑, productivity ↑, blood complement activity ↓ and C-reactive protein ↓ with no changes in other immune indicators, carcass quality ↑	(Suda et al., 2014)
	L. plantarum LP1	FCR ↓	(Jones et al., 2016)
Weaning	*L. paracasei*	*Clostridium* and *Enterobacteriaceae* ↓	(Nemcova et al., 1998)
	L. reuteri BSA131	ADG ↑, FCR ↑, enteric coliforms ↓	(Chang et al., 2001)
	L. gasseri, *L. acidophilus*, *L. fermentum*, *L. reuteri*	Growth performance ↑, *E. coli* infection ↓, microbial balance in the GIT ↑, diarrhea ↓	(Huang et al., 2004)
	L. reuteri I5007	Performance ↑	(Yu et al., 2008)
	L. reuteri I5007	ADG ↑, feed intake ↑, crude protein digestibility ↑, IgG level ↑, weaning stress syndrome ↓, antioxidative defense ↑	(Wang et al., 2009; Wang et al., 2012b; Wang et al., 2013)
	L. acidophilus C2, *L. plantarum* 1K8, *L. plantarum* 3K2	Feed intake ↑, ADG ↑, FCR ↓	(Giang et al., 2010)
	L. amylovorus	FCR ↓, Fatty acid profiles of pig meat ↑	(Ross et al., 2010; Ross et al., 2012)
	L. plantarum ATCC14917, *L. reuteri* DSM20016	BW ↑, ADG ↑	(Veizaj-Delia et al., 2010)

	L. rhamnosus GG	IL-6 produced by *E. coli* K88 ↓, diarrhea ↓	(Zhang et al., 2010)
	L. reuteri X-1	Growth performance ↑	(Wang et al., 2011)
	L. plantarum ZJ316	Growth performance ↑, pork quality ↑, gut morphology ↑	(Suo et al., 2012)
	L. acidophilus	Performance ↑	(Wang et al., 2012a)
	L. jensenii TL2937	Growth performance ↑, productivity ↑, carcass quality ↑	(Suda et al., 2014)
	L. reuteri, *L. plantarum*	Gross energy ↑, total tract digestibility of nitrogen ↑, lactobacilli ↑, *E. coli* ↓; fecal gas emission ↓, diarrhea ↓	(Zhao and Kim, 2015)
Growing	*L. plantarum* CJLP56	ADG ↑, bacterial fecal shedding ↓	(Gebru et al., 2010)
Growing-finishing	*L. reuteri* I5007	Total antioxidant capacity ↑, diarrhea ↓, T-cell differentiation ↑, cytokine expression in the ileum ↑	(Yu et al., 2008; Wang et al., 2009; Wang et al., 2012b; Wang et al., 2013)
	L. plantarum ZJ316	ADG ↑, FCR ↓, digestibility of crude protein and organic matter ↑, diarrhea ↓	(Suo et al., 2012)
Finishing	*L. plantarum* KCTC3624	Noxious gas emissions ↓, carcass weight and quality ↑	(Cha et al., 2015)
Sows	*L. johnsonii* XS4	Production performance ↑, serum IgG levels ↑	(Wang et al., 2014a)
	L. reuteri 3S7, *L. plantarum* 4-1	*Enterobacteriaceae* ↓, beta-glucuronidase activity ↓	(De Angelis et al., 2007)
P76 hybrid pigs	*L. casei* ATCC7469, *L. plantarum* ATCC8014	Meat quality ↑	(Rybarczyk et al., 2016)

* ↑ means upregulation or increase, ↓ means downregulation or decrease.

3) *Lactobacillus salivarius*

(1) General features of *L. salivarius*

The species *L. salivarius* owes its name to the 'salivary' properties of the oral cavity from which it was first isolated (Rogosa et al., 1953). The name thus acknowledges the intrinsic association of the species with the vertebrate GIT. *L. salivarius* is a Gram-positive bacterium that is an important member of the commensal bacterial communities of humans, swine and poultry, especially the intestinal microbiota (Heilig et al., 2002; Lan et al., 2002; Leser et al., 2002; Gong et al., 2007; Neville and O'Toole, 2010). The species is obligately homofermentative bacterium, which uses the Embden-Meyerhof-Parnas pathway for glycolysis and fermentation and produces lactate. *L. salivarius* has gained attention in recent years as a promising probiotic species (Dunne et al., 1999; Flynn et al., 2002; Sheil et al., 2004). Thus, this species and their probiotic action have to be considered in the context of a whole organism or living system. Of particular interest is the *L. salivarius* UCC118, and it is well-documented that this strain exhibits several probiotic traits (Thornton, 1996; Dunne et al., 1999).

(2) Probiotic properties of *L. salivarius*

There are many mechanisms which elicit enhanced intestinal health by probiotic bacteria. *L. salivarius* fulfills the principal requirements and properties of an efficient probiotic. Probiotic properties have been ascribed to many *L.*

salivarius strains such as the ability to modulate gut microbiota, produce antimicrobial substances, stimulate protective immune response, and produce SCFAs. Selection of a probiotic *L. salivarius* strain for *in vivo* applications included several *in vitro* characterization assays, such as assays of aggregation, co-aggregation, cell wall hydrophobicity, acid tolerance, bile salt tolerance, adhesion to epithelial cell lines, and antimicrobial activity (Dobson et al., 2012).

Antimicrobial activity *L. salivarius* have been focused on its ability of bacteriocin production, and many studies revealed the characters of the bacteriocins, termed salivaricin (Barrett et al., 2007; Pingitore et al., 2009; Messaoudi et al., 2011; O'Shea et al., 2011). The two-component class II bacteriocins (e.g. ABP-118, salivaricin CRL 1328 and salivaricin P) produced by *L. salivarius* strains isolated from mammals have been characterized at the molecular level (Flynn et al., 2002; Barrett et al., 2007; Pingitore et al., 2009). In addition, some bacteriocins (e.g. salivaricin FK22, OR7, L-1077 and SMXD51) produced by *L. salivarius* strains isolated from the chickens intestines are characterized by one-component class II bacteriocins (Pilasombut et al., 2006; Stern et al., 2006; Svetoch et al., 2011; Messaoudi et al., 2012b).

Antimicrobial activity by probiotic *L. salivarius* cultures has been demonstrated on several studies. Thus, Zhang and colleagues have isolated a number of *L. salivarius* strains from the chicken intestine, showed *in vitro* activity against *Salmonella* and *C. jejuni* (Zhang et al., 2011). Another study demonstrated

protection of mice with *L. salivarius* UCC118 against infection with the food-borne pathogen *L. monocytogenes* in mice (Corr et al., 2007). The observation that *L. salivarius* UCC118 survived in pig GIT and colonized to the pig ileum is concordant with *L. salivarius* DPC6005, which has been found to reduce *Salmonella* infection in pigs (Walsh et al., 2008). Moreover, these authors showed that among a five strain probiotic mixture, *L. salivarius* DPC6005 was predominantly recovered from ileal digesta and mucosa compared to the four other *Lactobacillus* species used in the mixture. It was suggested that this was due to a competitive advantage afforded by Salivaricin P produced by *L. salivarius* DPC6005 (Barrett et al., 2007).

Survival and adhesion in the GIT Resistant ability against acid and bile is generally considered as essential properties for probiotic evaluation since the strains have to survive the conditions in the stomach and the small intestine, and show ability to adhere and colonize the intestinal tract (Dunne et al., 2001). *L. salivarius* CECT5713 was strongly adhesive to Caco-2 and HT-29 cells (Maldonado et al., 2010). Consumption of the *L. salivarius* UCC118 by the children led to an increase in the fecal lactobacilli content, and this strain was detected in 90% of the feces of the volunteers, suggesting that the GIT. *L. salivarius* UCC118 survives in the pig GIT and adheres to the ileal mucosa (Corr et al., 2007). *L. salivarius* SMXD51 can tolerate GIT conditions and adhere to Caco-2/TC7 cells (Messaoudi et al., 2012a).

Stimulation of the immune system Many strains of *L. salivarius* demonstrate immunomodulatory activity. Administration of *L. salivarius* CECT5713 improved host immunity by inducing IL-10 and some Ig levels, as well as inducing an increase in the number of NK cell and monocyte (Pérez-Cano et al., 2010; Sierra et al., 2010). Another study showed that *L. salivarius* B1 increases the number of immuno-competent cells and enhances IL-6 expression in the pig intestine (Zhang et al., 2011). Several *L. salivarius* strains induced the production of IL-12 or IL-6 (O'Flaherty and Klaenhammer, 2010; Riboulet-Bisson et al., 2012). Messaoudi and colleagues showed that *L. salivarius* SMXD51 induces IL-8 secretion (Messaoudi et al., 2012a). Interestingly, *L. salivarius* SMXD51 in this study was also found to induce the secretion of the host defense peptide β-defensin 2, which displays a broad spectrum antimicrobial activity and plays an important role in the intestinal barrier function.

Modulation of the intestinal barrier Probiotics have been implicated as important mediators of intestinal barrier function and integrity. It was demonstrated that a probiotic mixture composed of *L. salivarius* W24 and other LAB trigger intestinal epithelial cells to synthesize Hsp70 (Malago et al., 2010). The increased level of Hsp70 repairs the mucosal integrity of Caco-2 cells after exposure to *S. enteritidis* 857. By stabilizing the actin filaments and the cytoskeleton, Hsps inhibit bacteria from adhering and invading the Caco-2 cells

(Mack et al., 2003). *L. salivarius* SMXD51 prevents the loss of barrier integrity provoked by *P. aeruginosa*, which has been shown to have a strong cytotoxic activity toward Caco-2/TC7 cells and to alter the F-actin cytoskeleton (Madi et al., 2010; Messaoudi et al., 2012a). It was also found that *L. salivarius* strains are widely diverse in their capacity for barrier protection, and this is underpinned by differences in the activation of intracellular signaling pathways. *L. salivarius* UCC118 and CCUG38008 protected the barrier function in Caco-2 cell by inducing phosphorylation of extracellular signal-regulated kinase (Miyauchi et al., 2012).

4) *Lactobacillus reuteri*

(1) General features of *L. reuteri*

This Gram-positive bacterium stably inhabits the GIT of mammals such as humans, pigs, hamsters, mice, rats, dogs, sheep and cattle as well as several species of birds (Casas and Dobrogosz, 2000; Reuter, 2001; Holzapfel and Wood, 2012). Moreover, *L. reuteri* is one of few lactobacilli that has been shown to be indigenous to humans, and has been isolated from infants and adults at various body sites including the gut, vagina, feces and human breast milk (Reuter, 2001; Abrahamsson et al., 2009). The species is therefore distinct from most lactobacilli found in the human intestine, which are strangers derived from fermented food, food of plant origin, and meat products (Walter, 2008). In some host species such as pigs, rodents and chickens, *L. reuteri* is present at high numbers and can be detected in a large subset of animals (Leser et al., 2002; Salzman et al., 2002; Brooks et al., 2003; Hilmi et al., 2007).

Belonging to the obligately heterofermentative group, *L. reuteri* uses the phosphoketolase pathway for fermentation of carbohydrates to lactate, acetic acid, ethanol and carbon dioxide. However, this pathway has poor energetic yields, and this is compensated by the ability of *L. reuteri* to use external electron acceptors to gain additional energy and increase growth rate (Sobko et al., 2005; Gerez et al., 2008). *L. reuteri* satisfies its growth requirements by inhabiting the animal proximal gut, where easily accessible nutrients are abundant.

(2) Probiotic properties of *L. reuteri*

L. reuteri is a gut symbiont benefiting its host as many studies have shown that strains of this species confers health benefits to both humans and animals. Beneficial attributes of *L. reuteri* have been researched intensively during the past several decades because of the common use as probiotics. Some beneficial characteristics of *L. reuteri* include protection against enteric infections, biosynthesis of B-complex vitamins, production of essential amino acids, modulation of host immunity, and prevention diseases such as IBD and neonatal NEC (Spinler et al., 2008; Saulnier et al., 2011; Walter et al., 2011; Liu et al., 2012; Liu et al., 2013).

Reuterin and antimicrobial activity Horizontal acquisition of *pdu-cbi-cob-hem* gene cluster (Morita et al., 2008; Santos et al., 2008) has enabled human strains to utilize 1,2-propanediol as an energy source (Sriramulu et al., 2008), and this is likely to be important colonization factor in the animal gut. This gene cluster is also responsible for cobalamin (vitamin B_{12}) biosynthesis and glycerol utilization as well as reuterin production (Talarico et al., 1988; Talarico et al., 1990; Morita et al., 2008; Sriramulu et al., 2008). Cobalamin has been identified to be an important colonization for some colonic bacteria (Goodman et al., 2009). Reuterin is a broad-spectrum antimicrobial compound, and increases fitness of *L. reuteri* by inhibiting growth of other microbes in the same niche (Walter et al., 2011). Reuterin is an effective agent against Gram-positive and Gram-negative

bacteria as well as yeasts, fungi and protozoa (Talarico et al., 1988; Spinler et al., 2008). Several evidence suggest that this compound exerts antimicrobial activity by inducing oxidative stress to other microbes (Schaefer et al., 2010), thus it contributes to colonization resistance against enteric bacterial pathogens (Spinler et al., 2008). *L. reuteri* strains are much more resistant to reuterin compared to other intestinal bacteria (Cleusix et al., 2007).

The ability of *L. reuteri* to antagonize other members of the gut microbiota is not limited to secrete reuterin. Heterofermentation of sugars yields lactic acid and acetic acid that lower the pH and therefore inhibit the growth of pathogens (Lindgren and Dobrogosz, 1990; Servin, 2004). This species has also been shown to produce hydrogen peroxide and bacteriocins (Toba et al., 1991). Administration of *L. reuteri* reduced growth of pathogenic bacteria and decreased mortality of chickens and turkeys during *Salmonella* and *E. coli* challenge, and these protective effects can be explained by increased competitive exclusion (Casas and Dobrogosz, 2000).

Immunomodulation *L. reuteri* has been shown to interact with the host immune system and may therefore play an important role in the maintenance of mucosal homeostasis and tolerance in the gut. This species promotes immune homeostasis by modulating APCs (Lin et al., 2008; Jones and Versalovic, 2009; Thomas et al., 2012), suppressing pro-inflammatory cytokines and inducing of regulatory T-cells (Karimi et al., 2009; Livingston et al., 2010; Liu et al., 2013).

In vitro assays revealed that *L. reuteri* DSM12246 is capable of inhibiting

inflammatory cytokines, such as IL-12, IL-6 and TNF-α (Christensen et al., 2002). Down-regulation of proinflammatory cytokines by *L. reuteri* strains have also been reported in macrophages and monocytoid cells from children with Crohn's disease (Peña et al., 2005; Lin et al., 2008; Jones and Versalovic, 2009). Mechanistic studies in human monocytoid cells revealed that certain human-derived strains are capable of converting L-histidine to histamine, which potently inhibits TNF-α production by signaling through histamine H2 receptor (Thomas et al., 2012). A study showed that *L. reuteri* strains significantly reduce the intestinal levels of IL-8 (Liu et al., 2010). The differential effects of *L. reuteri* strains on inflammation implicate a potential interaction between *L. reuteri* and immune cells in the intestinal mucosa. *L. reuteri* modulates dendritic cell function to promote development of regulatory T cells which produced increased levels of IL-10 and were capable of inhibiting proliferation of T cells (Smits et al., 2005; Hoffmann et al., 2008) (Hoffmann, et al., 2008). Additionally, colonization with *L. reuteri* 100-23 stimulated the development and increase of regulatory T cells (Livingston et al., 2010).

Beneficial immunoregulatory effects of *L. reuteri* have been successfully demonstrated in numerous animal models of chronic inflammation. *L. reuteri* is highly effective in reducing inflammation in several experimental models of colitis (Møller et al., 2005; Peña et al., 2005; Schreiber et al., 2009), and ameliorating disease *in vivo* due to enterohemorrhagic *E. coli* (Eaton et al., 2011). *L. reuteri* strains DSM17938 and MM2-3 significantly increased survival rate of the host and reduced the NEC via modulation of TLR4 and NF-κB signaling in

the intestine (Liu et al., 2012). *L. reuteri* ATCC55730 improves symptoms of infantile colic, diarrhea, atopic dermatitis, chronic constipation and gastrointestinal infections (Weizman et al., 2005; Coccorullo et al., 2010; Miniello et al., 2010). Collectively, these findings suggest that the modulation of the host immune system by *L. reuteri* may be important feature in the establishment and maintenance of symbiotic partnership.

Study 1. Comparative Genomics of *Lactobacillus salivarius* Focusing on Host Adaptation and Influence of the Ban of AGPs

1. Introduction

L. salivarius is a Gram-positive bacterium that is an important member of the commensal bacterial communities of humans, swine and poultry, especially the intestinal microbiota (Heilig et al., 2002; Lan et al., 2002; Leser et al., 2002; Gong et al., 2007). This lactic acid-producing bacterium is a promising probiotic candidate that displays resistance to acid and bile (Dunne et al., 1999; Fang et al., 2009), adherence to mucus of vertebrates (van Pijkeren et al., 2006), and bile salt hydrolase activity (Fang et al., 2009). Furthermore, this bacterium protects hosts against pathogenic infections through various mechanisms, including antimicrobial activity against pathogens (Flynn et al., 2002; Corr et al., 2007; Messaoudi et al., 2013), reduction of pathogen adhesion (O'Hara et al., 2006) and effects on host immune cells (O'Mahony et al., 2006).

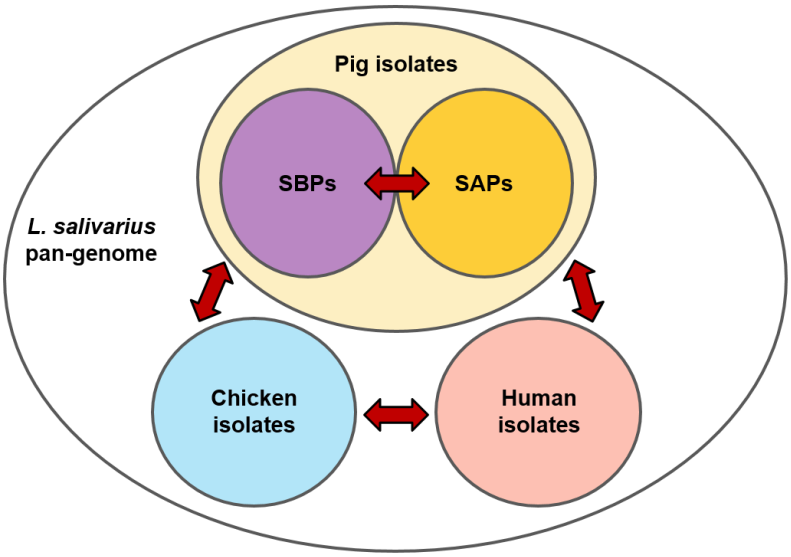
The clear relationship between the genomic diversity of lactobacilli and their hosts suggests that host adaptation drives the diversification and evolution of many *Lactobacillus* species, such as *L. reuteri*, *L. plantarum*, *L. casei*, *L. rhamnosus* and *L. acidophilus* (Berger et al., 2007; Cai et al., 2009; Oh et al., 2010;

Siezen et al., 2010; Douillard et al., 2013). Despite the importance of *L. salivarius* in animal intestines and the increasing use of this species as a probiotic, the majority of research on *L. salivarius* focuses on one strain, *L. salivarius* UCC118, which was isolated from the human intestine (Dunne et al., 1999). The previous studies did not observe the breadth of *L. salivarius* genomic diversity. To understand the genetic diversity of *L. salivarius* strains that reside in different habitats at the whole genome level, the draft genomes of 21 *L. salivarius* strains isolated from pigs were generated, and the pan-genome of *L. salivarius* was investigated using a comparative genomic approach that focused on properties contributing to host adaptation (Figure 9).

Furthermore, the influence of AGP prohibition on *L. salivarius* was observed in terms of evolutionary aspect (Figure 9). A persistent treatment of antibiotics such as AGPs in feeds has an impact on the intestinal bacteria of animals and their habitats. The intestinal circumstance has been changed considering several studies which proved the effect of antibiotics to intestinal microbiota (Antonopoulos et al., 2009; Greenwood et al., 2014; Panda et al., 2014). Moreover, many researches revealed that the use of antibiotics leads the evolution of bacteria (Balaban et al., 2004; Bush et al., 2011; Lázár et al., 2013). The change of bacterial strategies that overcome antibiotic stress was occurred rapidly and extensively with genetic mutations (Fridman et al., 2014). On the contrary to this, studies about evolutionary evidence of bacteria after the ban of AGPs have the limited criteria such as the decrease of AR of commensal bacteria (Boerlin et al., 2001; Aarestrup,

2003). This study provides valuable insights into the niche adaptation and evolution of *L. salivarius* strains, and offers the detail for probiotic properties of the commensal *L. salivarius* isolates from various host species.

A



B

Study	Category	Item
Pan-genome of *L. salivarius* - Host adaptation	General features	General genomic features
		Host origins
	Adhesion and colonization	Host-specific extracellular proteins
		Cell wall proteins
		EPS
		SecA2-SecY2 system
	Nutrient utilization	Pig-specific carbohydrate metabolism
		Chicken-specific carbohydrate metabolism
	Avoiding threats caused from hosts	Bile salt hydrolase
	Other features	Surface exculsion protein
		Exodeoxyribonuclease
		AR genes
Influence of the ban of AGPs - Genomic comparison of SBPs and SAPs	General features	Comparison with NCBI genomes
		Clustering SBPs and SAPs
	Functional difference	AR genes
		Difference in gene category
	Cell wall components	EPS
		Cell wall proteins
	In vitro validation	Biofilm formation
		Survival rate in competition

Figure 9. Graphical abstract of study 1.

(A) Organization of study 1. (B) Items observed in study 1.

2. Materials and Methods

1) Feces sampling and bacterial isolation

Porcine feces were collected from several swine farms following the policy and regulations for the care and use of laboratory animals (Laboratory Animal Center, Seoul National University, Korea). All of the experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Seoul National University (SNU-140522-3) and Kangwon National University (KW-140509-1).

Fecal samples were immediately collected in MRS broth (BD, NJ, USA) supplemented with 15% glycerol to preserve live LAB. The fecal samples were streaked on MRS agar (BD, NJ, USA) and incubated at 37°C for 24 h to isolate LAB.

2) Bacterial identification

LAB gDNA was extracted according to Reyes-Escogido *et al*. (Reyes-Escogido et al., 2010) with some modifications. Isolated bacteria were inoculated in MRS broth (BD, NJ, USA) and incubated at 37°C for 24 h. 400 μl of bacterial culture was collected, and cells were harvested by centrifugation at 8,000 g for 1 min. Cell pellets were washed with 1 ml TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0). The cells were resuspended in 200 μl TE buffer, and placed in a microwave oven for 1 min at 625 W, followed by cooling down for 30 sec at room temperature, and microwaved again under the same conditions. The lysates were briefly vortexed and centrifuged, and 100 μl supernatant containing the gDNA was recovered and quantified.

RAPD PCR was carried out with the bacterial gDNA. 40 ng of the purified gDNA was added to 20 μl of a PCR master mix containing 10 μl of i-Taq 2x PCR master mix solution (Intron Biotechnology, Seongnam, Korea), and a primer OLPII (5'-ACGATGAGCC-3'). PCR was performed as follows: 94°C for 5 min; 40 cycles of 93°C for 1 min, 37°C for 1.5 min, 72°C for 1 min; 72°C for 8 min.

For multiplex PCR, 20 ng of the purified gDNA was added to 20 μl of a PCR master mix containing 10 μl of i-Taq 2x PCR master mix solution (Intron Biotechnology, Seongnam, Korea), and primer sets (10 pmole per each primer); For *L. salivarius*, Lsal1 (5'-AATCGCTAAACTCATAACCT-3') and Lsal2 (5'-CACTCTCTTTGGCTAATCTT-3'); For *L. reuteri*, Lreu1 (5'-

CAGACAATCTTTGATTGTTTAG-3') and Lreu4 (5'-GCTTGTTGGTTTGGGCTCTTC-3') (Song et al., 2000). PCR was performed as follows: 94°C for 3 min; 35 cycles of 94°C for 20 sec, 60°C for 2 min; 72°C for 5 min.

3) Generation of draft genome of *L. salivarius* strains

Isolated *L. salivarius* strains were cultured in MRS broth for 24 h at 37°C and the bacterial cells were harvested. *L. salivarius* gDNA was extracted using G-spin Total DNA Extraction Kit (Intron Biotechnology, Seongnam, Korea) according to a standard protocol. A gDNA library for Illumina sequencing was constructed with ~350 bp inserts using Nextera XT DNA Library Preparation Kit (Illumina, CA, USA) according to the manufacturer's recommendations. The prepared *L. salivarius* gDNA libraries were then sequenced using Illumina HiSeq 2500 for 100 bp paired-end reads.

After the sequencing, adapter sequences of the reads were trimmed with Cutadapt 1.10 (Martin, 2011) and the sequence reads were quality-filtered using in-house Perl scripts (Kopit et al., 2014). In brief, when 95% of the nucleotide bases in a read were given a quality score over 31 (Illumina 1.8+) and the read length was ≥ 70 bp, the read was used for *de novo* genome assembly. The filtered paired reads were assembled using SPAdes 3.9 (Bankevich et al., 2012). ANIb, indicative of the relationships among species, was calculated by JSpeciesWS (Richter et al., 2016). All of the genome sequences of *L. salivarius* strains obtained were deposited into NCBI Whole Genome Shotgun database.

To test clonality between the generated genomes, the nucleotide sequences of the polymorphic sites in the core genomes were collected and compared. The degree of completion of the generated genomes was tested by aligning the

sequencing reads to the draft genome. Alignment of the short paired reads was carried out using Bowtie2 with the default options and portion of the mapped reads per total reads was represented (Langmead and Salzberg, 2012).

4) Genome collection and ortholog identification

All the *L. salivarius* genomes available in the NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/genomes/1207) in February of 2017 were collected for an investigation of the *L. salivarius* pan-genome (

Table 3). Although *L. salivarius* ATCC11741 and DSM20555 are identical strains, each has a genome sequence in the NCBI database. The more complete genome, ACGT00000000 consisted of 32 scaffolds, was selected instead of AYYT00000000 consisted of 62 scaffolds for further analyses. The 35 genomes were annotated RAST with the default options (Overbeek et al., 2014).

For the identification of orthologous CDSs, all CDSs of the 35 *L. salivarius* genomes were collected, and orthologs were identified as previously described (Figure 10) (Kim and Marco, 2014). Briefly, CDSs in the annotations were filtered to remove those containing premature stop codons (pseudogenes). Each CDS was then aligned to the entire CDS pool using GASSST 1.28, according to nucleotide sequence identity (≥ 85%) and maximum sensitivity (Rizk and Lavenier, 2010). The aligned CDSs were regarded as one ortholog, and the consensus sequence of each ortholog was determined using the CAP3 program with the default options (Huang and Madan, 1999).

Table 3. *L. salivarius* genomes obtained from NCBI database for this study.

Strain	Isolation host	Geographical origin	Genome size (Mb)	GC%	No. of CDSs	NCBI accession
CICC23174	Chicken	China	2.08	32.85	2,007	NZ_CP017107
NIAS840	Chicken	Korea	2.05	33.00	1,980	AFMN00000000
SMXD51	Chicken	Tunisia	1.97	32.97	1,900	AICL00000000
609_LSAL	Human	USA	1.84	32.70	1,803	JVAF00000000
778_LSAL	Human	USA	1.93	32.70	1,852	JUTI00000000
866_LSAL	Human	USA	1.97	32.70	1,903	JUQA00000000
ACS-116-V-Col5a	Human	N.D.	2.04	32.70	2,066	AEBA00000000
ATCC11741	Human	N.D.	2.02	32.70	1,963	ACGT00000000
CECT5713	Human	Spain	2.14	33.01	2,181	NC_017481
GJ-24	Human	Korea	2.00	33.00	1,944	AFOI00000000
Ren	Human	China	1.98	33.08	1,970	NZ_CP011403
UCC118	Human	Ireland	2.13	33.01	2,182	NC_007929
cp400	Pig	UK	2.16	32.90	2,179	CBVR00000000
JCM1046	Pig	Japan	2.32	32.97	2,313	NZ_CP007646

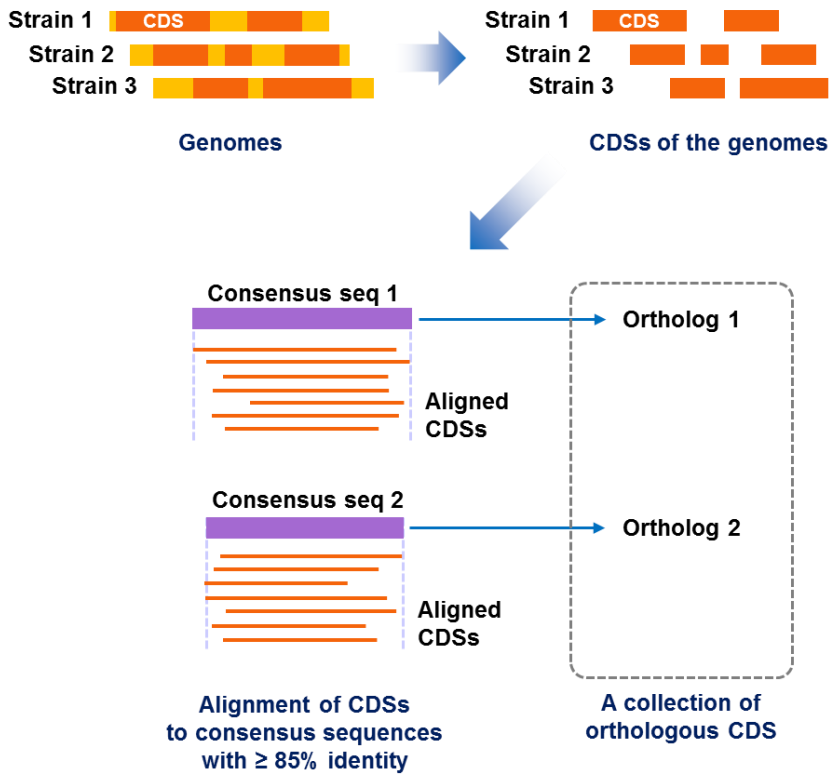


Figure 10. A method for construction of ortholog collection.

CDSs of the microbial genomes were collected and the CDSs were aligned to other CDSs. After the consensus sequence was determined, the CDSs aligned to one consensus sequence were regarded as the same ortholog. The figure was modified from Kim and Marco (2014).

5) Hierarchical clustering and functional analysis of genes

The presence of orthologs in a genome was used for hierarchical clustering using the Euclidean distance method implemented in R software (Ihaka and Gentleman, 1996). Existence of orthologs was statistically examined by 1,000 bootstraps using an R package, Pvclust (Suzuki and Shimodaira, 2006). Phylogenetic analyses based on nucleotide sequences were carried out using MEGA7 (Kumar et al., 2016). The nucleotide sequences were retrieved through the global alignment of orthologous CDSs from each genome and were compared using the multiple sequence alignment software MUSCLE 3.8.31 (Edgar, 2004). The phylogenetic relationship was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa being analyzed (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Nucleotide sequences of the seven *L. salivarius* housekeeping genes, *pstB, rpsB, pheS, ftsQ, nrdB, rpoA* and *parB* were used in clustering for MLSA (Raftis et al., 2011).

The assembled draft genome sequences were uploaded to RAST server with default options for bacteria to obtain information on gene functional categories, called subsystems (Overbeek et al., 2014); and the gene amounts were counted for each subsystem. Differentially over-represented gene numbers in each subsystem were examined between two groups of genomes using Fisher's exact test.

Cell wall-anchored proteins with a choline-binding domain (Pfam family: CW_binding), LPXTG domain (Gram_pos_anchor), lipoprotein anchor (Lipoprotein_Ltp), LysM domain (LysM), peptidoglycan-binding domain (PG_binding) and WXL domain (WXL) were identified for further analysis using the Pfam database (Finn et al., 2016). Mucus-binding protein (MucBP) encoding genes were also identified using Pfam. For analyses of genes associated with EPS production, 50 EPS-related genes of *L. salivarius* UCC118 were collected from a previous study (Raftis et al., 2011), and the pan-genome orthologs that belonged to the same Pfam family of EPS genes in UCC118 were obtained and filtered manually. Sequences of AR genes were obtained from the CARD (McArthur et al., 2013). Identification of ISs was carried out using ISfinder (Siguier et al., 2006). Orthologous CDSs of the *L. salivarius* genomes were aligned to AR genes using MUSCLE for detection of the genes in each genome (Edgar, 2004). Sequence identity was calculated by BLAST+ (Camacho et al., 2009) and each ortholog having E-value ≤ 0.0001 and identity $\geq 90\%$ was regarded as the identical gene in the list of the analyzed genes.

6) *In vitro* validation

Biofilm formation of *L. salivarius* strains was analyzed as described by Ambalam et al. (2012) with some modification (Figure 11A). Each well of sterile TPP flat-bottomed 96 well microplates was filled with 200 μl of MRS broth. Overall, ~2 × 10^6 *L. salivarius* cells were added to each well and incubated under static conditions at 37°C for 48 and 72 h. The plates were then washed twice with PBS and dried for fixation at 55°C for 20 min. All plates were washed three times with PBS and the bacteria attached to the surface were stained with 200 μl of 0.1% (w/v) crystal violet in 1 : 1 : 18 of isopropanol-methanol-PBS solution (v/v). Excess dye was rinsed off by washing three times with PBS. The residual dye bound to the surface-adhered cells was extracted with 200 μl of 30% glacial acetic acid, and the absorbance of each well was measured at 630 nm in a microplate reader (Infinite M200 Pro, Tecan, Zürich, Switzerland). The amount of surface-bound dye was determined using a standard curve for crystal violet (μg).

Survival rate of the *L. salivarius* in competition with swine intestinal microorganisms was also measured (Figure 11B). Fecal microbes were prepared according to a previous study with some modifications (Goodman et al., 2011). Feces were obtained from eight pigs that did not take any probiotics or antibiotics, and two grams of the mixed feces were placed in 30 ml of pre-reduced PBS. The fecal material was suspended by vortexing for 5 min, and the suspension was allowed to stand at room temperature for 10 min. Supernatant of the suspended feces sample was diluted, and ~5 × 10^7 fecal microbes were plated on each well

of 24-well plates containing 1.6 ml of pre-reduced BHI broth (BD, NJ, USA). *L. salivarius* strains were cultured in MRS broth for 24 h at 37°C and the cells were washed with PBS. 1×10^5 cells of each strain were added to each well of the fecal microbe-containing plates. They were incubated at 37°C for 24 and 48 h, and then the cells were harvested for *L. salivarius* quantification. All of the processes were carried out under anaerobic condition (an atmosphere of 75% N_2, 20% CO_2 and 5% H_2).

The amount of *L. salivarius* strains was calculated by real-time PCR with *L. salivarius*-specific primers, For-Sal-3 (5'-GTCGTAACAAGGTAGCCGTAGGA-3') and Rev-Sal-1 (5'-TAAACAAAGTATTCGATAAATGTACAGGTT-3') (Harrow et al., 2007). gDNA of the strains was extracted by microwaving as described above. Amplification reaction mixtures (total volume 20 μl) contained 40 ng of the extracted gDNA, 10 μl of SYBR green qPCR 2X premix (Enzynomics, Daejeon, Korea) and 500 nM of each primer. Tests were performed with CFX96 Touch Real-Time PCR Detection System (BioRad, CA, USA). PCR was performed as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 63°C for 1 min. To determine the specificity of the SYBR green PCR assay, a melting curve analysis of the DNA fragments was performed according to the manufacturer's instructions. Standards were used to determine the amount of *L. salivarius* DNA by real-time PCR using a modified protocol of a previous study (Byun et al., 2004). Purified gDNA ranging from 1 pg to 100 ng of *L. salivarius*

KLF003 was used as the standard. This was equivalent to approximately 4.16 × 10^2 to 4.16 × 10^7 copies of the genome (the average genome size of the strains used in this study is 2.21 Mbp).

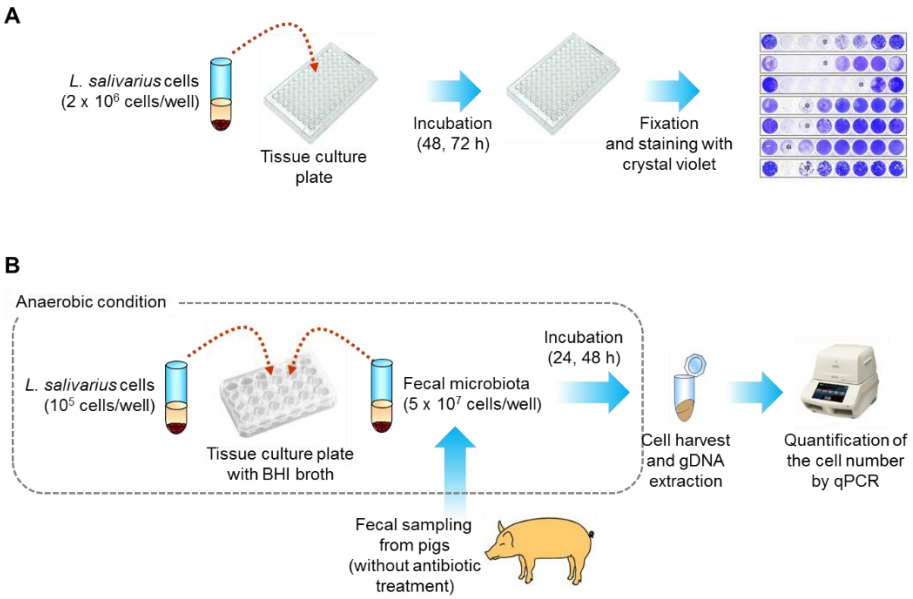


Figure 11. *In vitro* validation assays for study 1.

(A) Biofilm formation assay. *L. salivarius* cells were cultured in a tissue culture plate and biofilm was stained by crystal violet. (B) Competition assay with fecal microbiota. Fecal microbiota was obtained from fresh porcine feces and incubated with *L. salivarius* cells under anaerobic condition. The number of *L. salivarius* cells was counted by quantitative PCR.

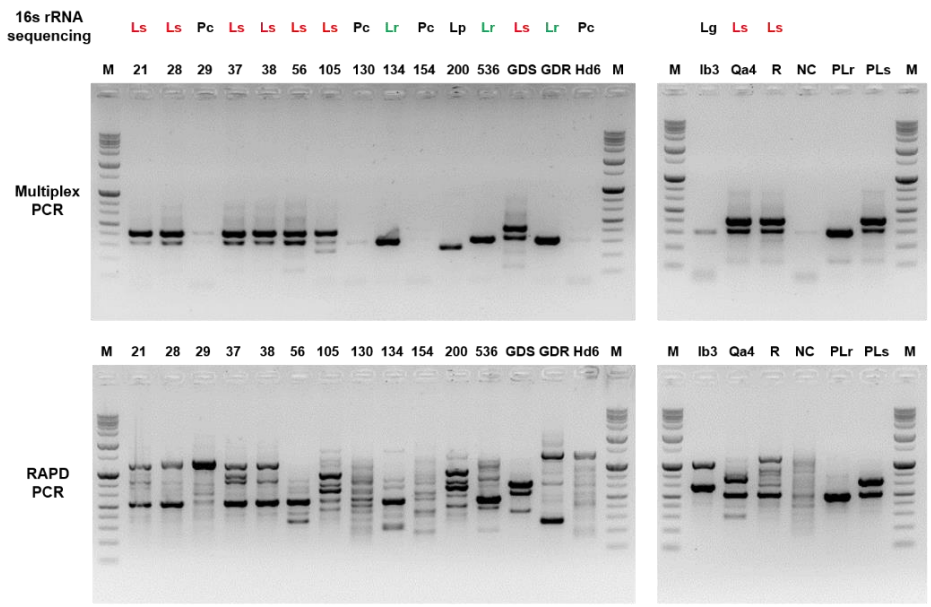
3. Results and Discussion

1) Isolation and identification of *L. salivarius* and *L. reuteri*

The *L. salivarius* and *L. reuteri* strains that inhabited the intestinal tracts of swine were isolated from several farms of Republic of Korea under two different conditions: 1) AGPs had been used as feed additives for livestock (in 2005 and 2006), and 2) after the ban on AGP addition to the feeds (in 2014 and 2015). 211 and 423 LAB colonies were isolated from porcine feces obtained before and after AGP prohibition, respectively.

Species identification of LAB colonies was carried out with RAPD PCR and species-specific multiplex PCR methods (Figure 12). A result of RAPD PCR showed the complex band patterns and these are not identical with the 16s rRNA sequencing. On the other hands, multiplex PCR result matched up with the 16s rRNA sequencing, therefore multiplex PCR was selected for identification of LAB colonies. The colonies were identified by species-specific PCR, and 284 of *L. salivarius* colonies and 104 of *L. reuteri* colonies were obtained among the 634 LAB colonies. *L. salivarius* colonies and *L. reuteri* colonies were used in study 1 and study 2, respectively.

A



B

Origin	Fecal samples	LAB isolates	*L. salivarius*	*L. reuteri*
Feces from finishing pig	70	247	119	51
Feces from weaning pig	80	176	76	22
Previously isolated colonies	-	211	89	31
Total		634	284	104

Study 1 Study 2

Figure 12. Bacterial identification for both study 1 and 2.

(A) Methods for bacterial identification. Multiplex PCR and RAPD PCR were compared with 16s rRNA gene sequencing. Ls, *L. salivarius*; Lr, *L. reuteri*; Pc, *P. clausseuii*, Lp, *L. plantarum*; Lg, *L. gasseri*; NC, negative control, PLr and PLs, positive controls of *L. reuteri* and *L. salivarius* obtained from KCTC. (B) The result of bacterial identification using the PCR method shown in panel A. *L. salivarius* and *L. reuteri* were used in study 1 and 2, respectively.

2) Pan-genome of *L. salivarius*

(1) General features of the *L. salivarius* genomes

The 284 *L. salivarius* colonies consisted of 89 and 195 colonies from before and after AGP prohibition, respectively. Among them, 21 *L. salivarius* strains including six strains isolated before AGP prohibition (SBPs) and fifteen strains isolated after AGP prohibition (SAPs) were randomly selected. Whole genome sequencing of SBPs and SAPs was carried out, and draft genomes of the strains were generated (Table 4). The degree of completion of draft genomes was measured by aligning the sequencing reads to the draft genome. All genomes reflect over 98% of the complete genomes except one genome (KLW007, 95.8%; mapped reads in Table 4). Clonality of the genomes was also tested by confirming polymorphic sites in the core genomes of the 21 *L. salivarius* strains. Total 37,828 polymorphic sites were found and the hierarchical clustering of the polymorphic sites showed the distinction of the 21 strains (Figure 13). This result indicated that the *L. salivarius* strains are genetically different from each other.

In addition to the 21 strains isolated in this study, 14 genomes were obtained from NCBI genome database. Total 35 genome sequences of *L. salivarius* strains, consisting of 23 pig isolates, 9 human isolates and 3 chicken isolates, were collected for this study (Table 3 and Table 4). Each draft genome was compared to the *L. salivarius* UCC118 reference genome by measuring the percentage of ANIb. All of the strains showed over 97% of ANIb values through pairwise

comparison with the reference genome of the species, *L. salivarius* UCC118, indicating that all of the strains used in this study belonged to the same species, *L. salivarius* (Richter and Rosselló-Móra, 2009).

The 35 *L. salivarius* strains have an average genome size of 2.14 ± 0.14 Mbp, 2062.74 ± 134.26 genes and a GC content of 32.84 ± 0.12%. The genome size and number of genes for the pig isolates were larger than those of the human and chicken strains (Table 5). However, GC content was not different between isolates in relation to their hosts. Orthologous CDSs of all available *L. salivarius* genomes were collected and used in hierarchical clustering. Two phylogenetic trees were constructed based on the ortholog presence or MLSA gene sequence (Figure 14). The phylogenetic clustering showed that *L. salivarius* strains are distinguished by the host from which they were isolated, not geographical origin, and this result was also observed for other *Lactobacillus* species, such as *L. reuteri*, *L. casei* and *L. rhamnosus* (Vancanneyt et al., 2006; Cai et al., 2007; Oh et al., 2010). The phylogenetic clustering using MLSA genes formed three distinct lineages based on the host-dependent clustering, indicating that *L. salivarius* strains from the same host have a common ancestor, except ATCC11741 and GJ-24.

L. salivarius ATCC11741 and GJ-24 were not clustered by their host origin in the MLSA, although these strains originated from humans (Rogosa et al., 1953; Cho et al., 2011), implying that the ancestors of ATCC11741 and GJ-24 would be commensal bacteria of chickens and pigs, respectively. Of note, ATCC11741 showed a similar ortholog distribution with the other human isolates, while GJ-24

clustered with the chicken isolates. This suggests that ATCC11741, but not GJ-24, had become completely specialized to the human habitat (Figure 14A). GJ-24 strains possessed 124 unique genes, which are much higher than the other assayed strains in the pan-genome (average of 29.85 unique genes per strain). The RAST annotation showed that the products of the unique genes of GJ-24 are analogous with those of the chicken isolates; GJ-24 possessed several genes that were not found in the other eight human isolates but were present in the chicken isolates (e.g. SecA2-SecY2 system). However, the genes of chicken isolates and the genes of GJ-24 were not designated as identical orthologs, because their nucleotide identities are under 85%. Indeed, GJ-24 is distant from the other strains in the phylogenetic tree based on the nucleotide sequence of secA2 (Figure 15). These results indicate that GJ-24 did not lose the genes that are analogous with the chicken isolates, but underwent rapid mutation under strong selection pressure to adapt to the human host, resulting in GJ-24 possessing several unique genes.

To gain a clear understanding of the host specificity of *L. salivarius* strains, strain GJ-24 was excluded from further analyses, and orthologs of the remaining 34 *L. salivarius* genomes were collected. There are 4,431 orthologs in the *L. salivarius* pan-genome, which contains 1,062 core genes and 1,015 unique genes (Figure 16). 15 host-specific genes and 16 dual-host-shared genes that the isolates from another host did not possess were also found (Figure 17 and Table 6). The *L. salivarius* pan-genome was investigated, focusing on the host-specific or dual-host-shared orthologs.

Table 4. *L. salivarius* draft genomes generated in this study.

Group	Strain	Sampling farm	Genome size (Mbp)	No. of contigs	Max. contig size (Kbp)	N50	No. of CDSs	GC%	ANIb* (%)	Read depth (fold)	Mapped reads (%)	NCBI accession
SBP	KLA001	A	2.27	109	136.06	37,518	2,171	32.83	97.16	273.28	99.60	LXZT00000000
SBP	KLA002	B	2.26	101	118.15	41,911	2,175	32.82	97.17	217.09	98.10	LXZS00000000
SBP	KLA003	C	2.26	115	131.03	37,566	2,169	32.83	97.17	225.21	98.89	LXZR00000000
SBP	KLA004	D	2.27	111	118.16	40,763	2,180	32.83	97.15	359.66	98.93	LXZQ00000000
SBP	KLA005	A	2.26	100	149.45	41,688	2,177	32.82	97.16	278.94	98.74	LXZP00000000
SBP	KLA006	E	2.37	167	126.01	37,519	2,265	32.93	97.15	255.61	98.85	LXZO00000000
SAP	KLF002	G	2.18	152	118.16	37,935	2,098	32.81	97.27	286.70	98.02	LXZM00000000
SAP	KLF003	G	2.21	124	121.00	36,200	2,128	32.73	97.34	335.31	98.30	LXZL00000000
SAP	KLF004	G	2.12	106	79.44	35,397	2,036	32.71	97.27	328.50	98.11	LXZK00000000
SAP	KLF005	F	2.15	126	108.15	33,039	2,020	32.69	97.03	359.68	98.86	LXZJ00000000
SAP	KLF007	F	2.22	115	135.06	44,101	2,138	32.90	97.19	244.36	98.76	LXZH00000000
SAP	KLW001	H	2.33	166	131.27	43,765	2,194	33.04	97.28	257.88	99.02	LXZG00000000
SAP	KLW002	H	2.09	107	146.33	44,394	1,982	32.82	97.47	439.92	99.20	LXZF00000000
SAP	KLW003	H	2.37	240	129.46	34,177	2,233	32.92	97.17	190.03	99.31	LXZE00000000
SAP	KLW004	H	2.34	247	105.40	27,732	2,185	32.82	97.16	372.00	98.58	LXZD00000000
SAP	KLW005	H	2.08	91	166.08	46,785	1,971	32.82	97.48	530.09	98.75	LXZC00000000
SAP	KLW006	F	2.03	100	134.07	36,518	1,892	32.70	97.17	366.12	99.06	LXZB00000000
SAP	KLW007	F	1.97	96	106.85	33,562	1,819	32.75	96.99	302.75	95.80	LXZA00000000
SAP	KLW008	F	2.14	163	146.65	38,328	2,011	32.88	97.42	275.62	98.91	LXYZ00000000
SAP	KLW009	F	2.07	86	160.11	50,465	1,961	32.86	97.46	252.02	98.22	LXYY00000000
SAP	KLW010	F	2.39	217	106.86	33,672	2,148	32.67	97.30	253.20	99.19	LXYX00000000

* ANIb value to the reference strain, *L. salivarius* UCC118.

Table 5. Averages of *L. salivarius* genomic features.

Host origin	Genome size (Mbp)	GC content (%)	No. of genes
Pigs	2.21 ± 0.12 [a]	32.83 ± 0.09	2106.30 ± 123.31 [a]
Humans	2.01 ± 0.09 [b]	32.84 ± 0.17	1984.89 ± 134.03 [b]
Chickens	2.03 ± 0.06 [b]	32.94 ± 0.08	1962.33 ± 55.64 [b]

Values were denoted by mean ± standard deviation. Averages followed by same letters in each column are not statistically different ($P < 0.05$).

Table 6. List of host-specific genes or dual-host-shared genes of *L. salivarius* pan-genome.

Host(s)	Ortholog_ID	Product	Locus tag
Pig			**in JCM1046**
	Ortholog_43	Nucleotide sugar synthetase-like protein	LSJ_0077
	Ortholog_102	MucBP mucin binding domain protein with YSIRK signal peptide	LSJ_0784
	Ortholog_310	Bsh2, Choloylglycine hydrolase (EC 3.5.1.24)	LSJ_0788
	Ortholog_1434	Glycosyltransferase (EC 2.4.1.-)	LSJ_0076
	Ortholog_2621	Hypothetical protein	LSJ_0074
	Ortholog_3201	Hypothetical protein	LSJ_0792c
	Ortholog_3862	Argininosuccinate lyase (EC 4.3.2.1)	LSJ_0791
	Ortholog_4063	MucBP	LSJ_0290
Human			**in UCC118**
	Ortholog_706	Cell surface protein precursor	LSL_0351
Chicken			**in CICC23174**
	Ortholog_2414	Hypothetical protein	BHF65_06140
	Ortholog_2329	Hexosephosphate transport protein, integral membrane protein	BHF65_02295
	Ortholog_3899	Hypothetical secreted protein	BHF65_00925
	Ortholog_799	Lactate 2-monooxygenase (EC 1.13.12.4)	BHF65_02290
	Ortholog_3186	Methylase (EC:2.1.1.-)	BHF65_05555

	Ortholog_2124	Surface exclusion protein Sea1/PrgA on plasmid pTEF2	BHF65_02330	
Pig and chicken			**in JCM1046**	**in CICC23174**
	Ortholog_116	Accessory secretory protein Asp2	LSJ_0059	BHF65_02225
	Ortholog_1338	Accessory secretory protein Asp1	LSJ_0058	BHF65_02230
	Ortholog_1786	Nucleotide sugar synthetase-like protein	LSJ_0071	BHF65_02165
	Ortholog_284	Protein export cytoplasm protein SecA2	LSJ_0061	BHF65_02215
	Ortholog_3638	Glycosyl transferase, family 8	LSJ_0073	BHF65_02160
	Ortholog_385	Accessory secretory protein Asp3	LSJ_0060	BHF65_02220
	Ortholog_3997	Preprotein translocase secY2	LSJ_0057	BHF65_02235
	Ortholog_4055	Nucleotide sugar synthetase-like protein	LSJ_0075	BHF65_02155
	Ortholog_1137	GftB, Glycosyl transferase, family 8	LSJ_0070	BHF65_02175
	Ortholog_2892	Glycosyltransferase (EC 2.4.1.-)	LSJ_0078	BHF65_02135
	Ortholog_4398	Glycosyl transferase, family 8	LSJ_0084	BHF65_21030
	Ortholog_2738	Surface protein SdrI, cell-wall-anchored protein (LPXTG motif)	LSJ_0063	BHF65_02210
	Ortholog_2246	Poly(glycerol-phosphate) alpha-glucosyltransferase GftA (EC 2.4.1.52)	LSJ_0068	BHF65_02195
Pig and human			**in JCM1046**	**in UCC118**
	Ortholog_401	XseB, exodeoxyribonuclease VII small subunit (EC 3.1.11.6)	LSJ_0581	LSL_0535
	Ortholog_3356	Hypothetical protein	LSJ_0984	LSL_0963

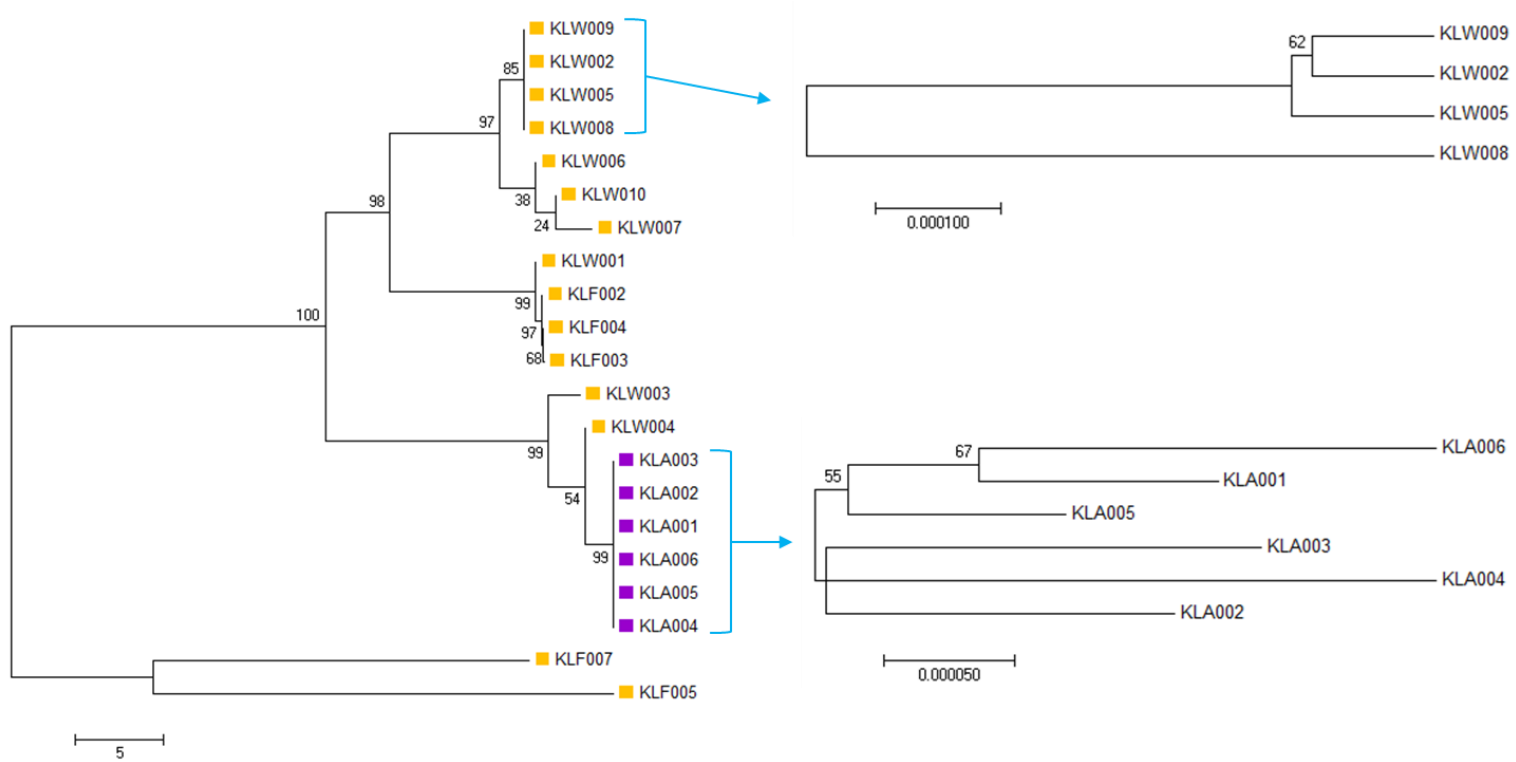


Figure 13. Hierarchical clustering of 21 *L. salivarius* genomes based on the polymorphic sites.

The genomes generated in this study were clustered based on the nucleotide sequence of the polymorphic sites in the core genomes. Purple box, SBP; yellow box, SAP.

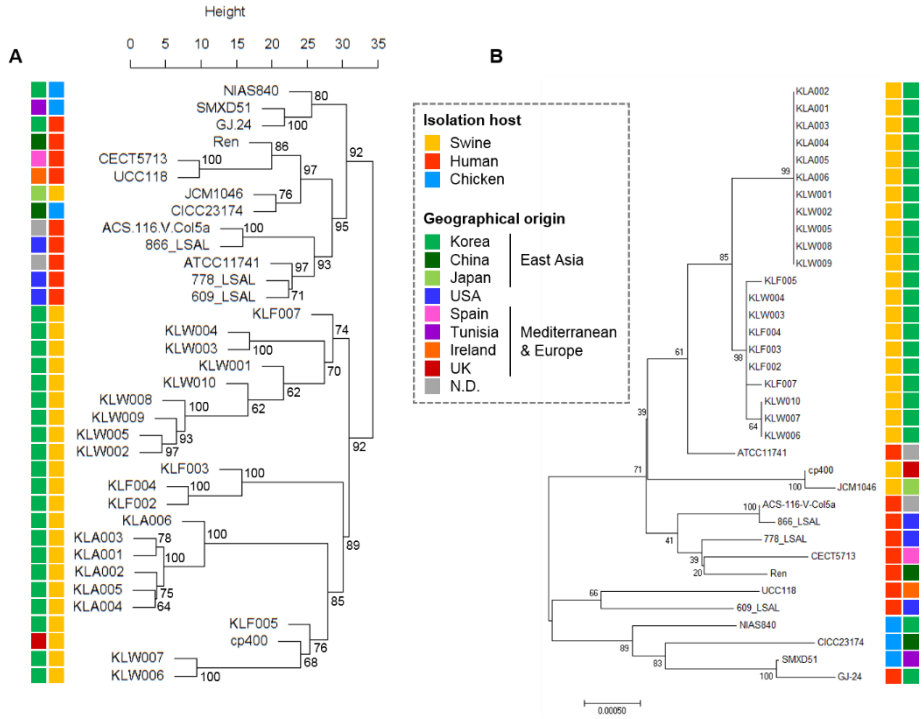


Figure 14. Hierarchical clustering of *L. salivarius* strains.

The clustering was carried out based on the ortholog presence (A) and nucleotide sequence of the MLSA genes (B). The bootstrap consensus tree inferred from 1,000 replicates is taken.

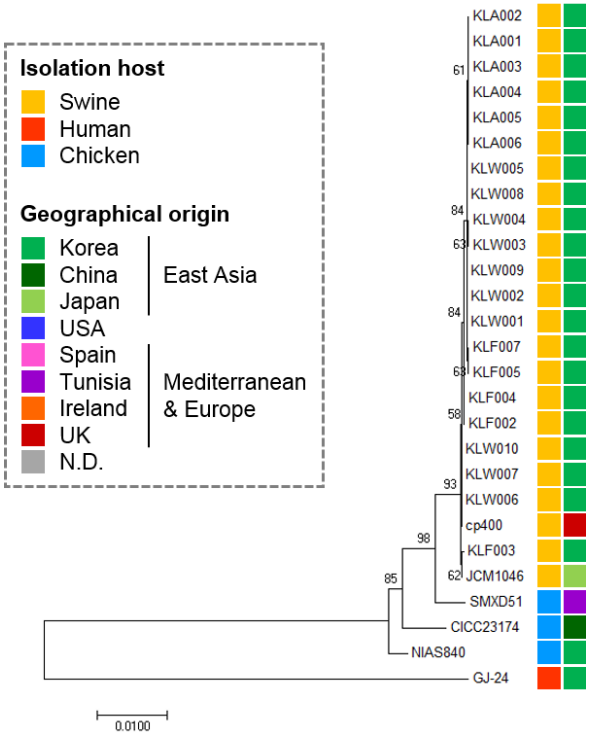


Figure 15. Phylogenetic clustering of 27 *L. salivarius* strains based on nucleotide sequence of *secA2* homologs.

The bootstrap consensus tree inferred from 1,000 replicates is taken.

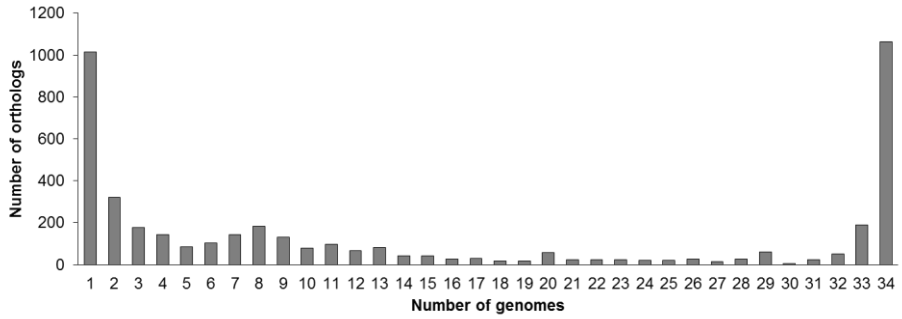


Figure 16. Distribution of orthologous CDSs in *L. salivarius* pan-genome.

Distribution histograms are shown for 34 *L. salivarius* genomes used in this study. The horizontal axis indicates the number of isolates sharing the same orthologous CDS, and vertical axis represents the number of orthologous CDS shared by the indicated number of isolates. When number of genomes is 34, number of orthologs means the number of core genes.

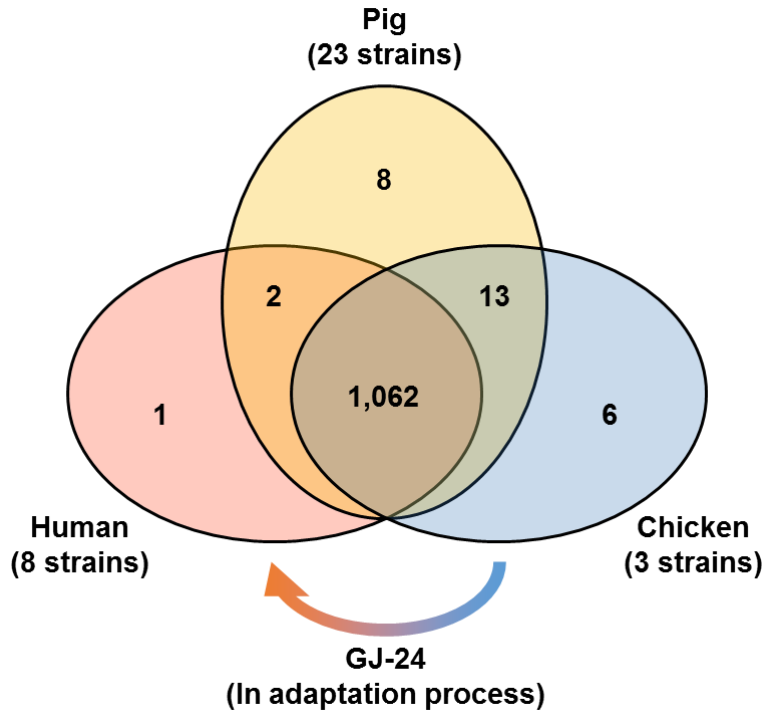


Figure 17. Pan-genome of the *L. salivarius* strains.

Genomes isolated from each host are represented by a colored circle. The number in the center represents the core genes shared among the genomes expressed as orthologous gene clusters of genomes from a same host. The numbers of genes unique to each host are indicated by the outer values. GJ-24 was excluded from the circles.

(2) Abilities for adhesion and colonization

Host-specific extracellular proteins

Bacteria acquire or lose genes during evolution to increase their fitness within habitats (Ochman and Moran, 2001; Toft and Andersson, 2010), and the majority of bacterial host adaptation is derived through HGT. During this process, essential genes for survival in a certain habitat would have been conserved among the isolates from each host. Among the 10 annotated and host-specific genes, 40% encode extracellular proteins, including both surface-anchored and secreted proteins (Table 6), suggesting that proteins related to the cell surface are important to host specialization. Indeed, several studies had revealed that extracellular proteins play an important role in many essential interactions and adaptations of LAB to their habitats (Lebeer et al., 2008; Kleerebezem and Vaughan, 2009), although the adhesion-related properties of lactobacilli varies between strains or species (Jacobsen et al., 1999; Jonsson et al., 2001). Therefore, an investigation of host-specific bacterial genes for extracellular proteins is a key to understanding host adaptation of *L. salivarius*.

Among the eight pig isolate-specific genes in the pan-genome, ortholog_102 and ortholog_4063 encode membrane proteins that facilitate bacterial adhesion to a host habitat (Table 6). Ortholog_102 encodes a protein containing a membrane anchor domain for Gram-positive bacteria and ortholog_4063 encodes a MucBP. The human and chicken isolates also possess host-specific genes for extracellular

proteins. The human isolates have a gene encoding a cell surface protein precursor (ortholog_706) and the chicken strains possess a gene for a hypothetical secreted protein (ortholog_3899). The Pfam annotation predicted that a protein encoded by ortholog_706 has a DUF3324. This domain has no characterized function, but it is reported that proteins which have DUF are not essential. It is believed that many DUF-containing proteins are required only under certain conditions (Häuser et al., 2012). It is predicted that ortholog_3899 encodes a 107 amino acid secreted protein (not a transmembrane protein) with a 27 amino acid signal peptide on its N-terminus. However, the evidence is not found that ortholog_706 and ortholog_3899 help in host adaptation of *L. salivarius* strains. Furthermore, among seven MucBP genes found in the pan-genome, there is evidence for host specialization in only one, ortholog_102 (Figure 18), suggesting that other factors for adhesion to host environments are present.

A large proportion of host specific genes for niche adaptation appear to be acquired from other microbes as there is evidence of HGT, including the presence of the transposase genes and ISs near to host-specific genes in the genomes. In the genome of the pig isolate JCM1046, several transposase genes are located in the flanking regions of ortholog_102 and ortholog_4063, both of which facilitates colonization to the pig intestine (LSJ_0782, LSJ_0783 and LSJ_0787 adjacent to ortholog_102 (LSJ_0784); LSJ_0297 and LSJ_0298 adjacent to ortholog_4063 (LSJ_0290)). Moreover, many ISs were identified within 5 kb of both the flanking regions of ortholog_102 and ortholog_4063 (Table 7), and ISs nearby host-

specific genes were also found in other lactobacilli, such as *L. reuteri*, *L. helveticus* and *L. bulgaricus* (Callanan et al., 2008; Morita et al., 2008; Liu et al., 2009). The chicken isolate CICC23174 also possesses two transposase genes (BHF65_00920 and BHF65_00915) within the flanking regions of ortholog_3899 (BHF65_00925), and ISs were found within the flanking regions of ortholog_706 in UCC118 and for ortholog_3899 in CICC23174 (Table 7), suggesting that these two human or chicken isolate-specific orthologs are associated with host adaptation.

Table 7. ISs within 10 kb of flanking region of extracellular protein genes.

Ortholog_ID	IS family	Number of IS
Ortholog_102 (LSJ_0784 in JCM1046)	ISL3	6
	IS3	2
	IS110	1
	IS5	2
	IS1634	1
	IS30	2
	IS1595	2
	ISNCY	1
	IS1380	1
	Tn3	2
	Total	20
Ortholog_706 (LSL_0351 in UCC118)	IS1380	2
	IS21	1
	IS1595	2
	IS1	2
	ISNCY	1
	IS481	1
	ISAs1	2
	IS3	2
	IS200/IS605	1
	IS256	1
	Total	15

Ortholog_ID	IS family	Number of IS
Ortholog_4063 (LSJ_0290 in JCM1046)	IS5	5
	IS21	1
	IS4	5
	IS91	1
	IS110	2
	IS1595	1
	IS607	1
	IS1634	3
	Tn3	1
	IS30	1
	Total	21
Ortholog_3389 (BHF65_00925 in CICC23174)	IS200/IS605	8
	IS1595	2
	IS21	1
	IS607	1
	ISNCY	1
	ISKra4	1
	IS1182	2
	ISAs1	1
	IS3	2
	IS30	1
	IS256	1
	Total	21

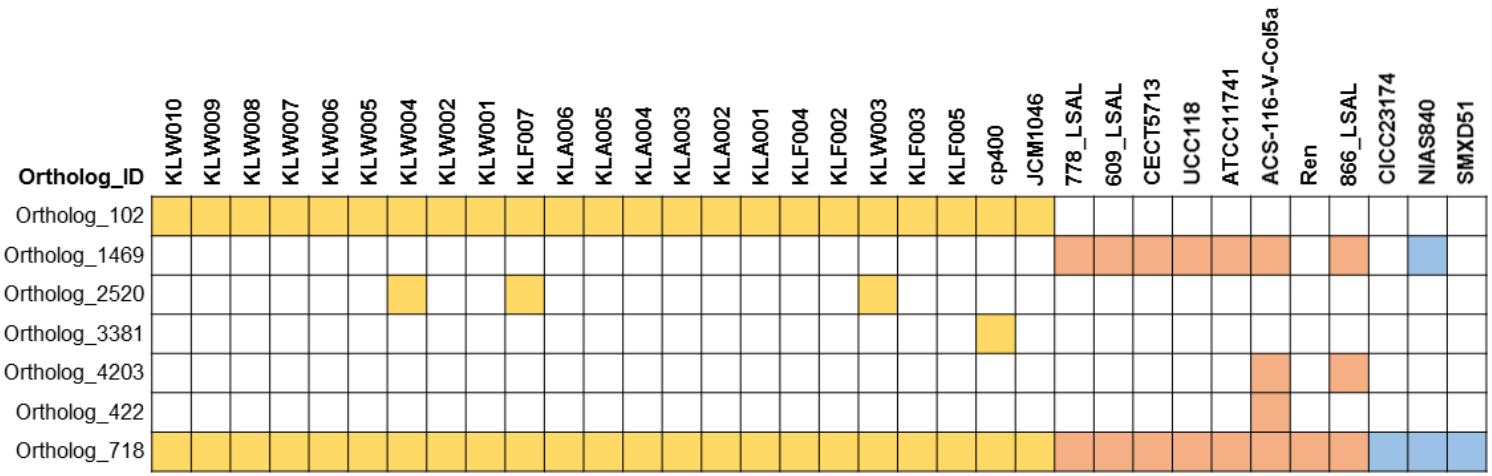


Figure 18. Presence of the genes encoding MucBPs associated with adhesion property.

Colored box means gene presence in the genome. Yellow box, pig isolates; red box, human isolates; blue box, chicken isolates. Ortholog_102, ortholog_1469, ortholog_2520, ortholog_3381 and ortholog_4203 encode proteins with LPXTG motif, and used in Figure 19.

Genes for cell wall components

It is known that various extracellular components of bacteria are responsible for niche adaptation through various interactions with hosts. Therefore, all the extracellular protein encoding genes within the *L. salivarius* pan-genome were determined. In total, 56 orthologs encoding 11 proteins with a LysM domain, 2 WXL domain proteins, 11 choline-binding proteins, 28 SDPs with a LPXTG motif, 3 proteins with a lipoprotein anchor and 1 peptidoglycan-binding protein were identified. Hierarchical clustering based on the presence of the 56 orthologs showed that the bacteria were grouped by the hosts from which they were isolated (Figure 19). Furthermore, a comparison of the nucleotide sequences of eight core genes among the extracellular protein encoding genes revealed that those of chicken isolates are genetically distinct from those of the human and pig isolates (Figure 20A), reflecting the differences between the bacterial habitats, mammals and birds. This host specialization of the extracellular protein encoding genes suggests that many extracellular proteins are responsible for host adaptation despite not being host-specific and that under selection pressures, host adaptation have been driven not only by HGT but also by mutation. It is notable that *L. salivarius* has developed abilities for adaptation during the evolutionary process, and this host adaptation from genomic changes is found in many bacteria (Cai et al., 2009; Oh et al., 2010; Toft and Andersson, 2010).

In Gram-positive bacteria, EPSs are important membrane components for niche adaptation and colonization, in addition to extracellular proteins (Vélez et al.,

2007). A previous study showed that *L. salivarius* possesses several EPS genes and that this species displays a high level of genomic diversity in EPS gene clusters between several strains (Raftis et al., 2011). Because this study had used the EPS genes of UCC118 as a reference, genes associated with EPS production in *L. salivarius* pan-genome were newly identified, and confirmed the genetic variation of these genes. A total of 124 orthologs associated with EPS production were collected, including 50 genes used in the previous study. The bacterial strains did not group by the distribution of EPS genes (Figure 21), but the phylogenetic clustering based on the nucleotide sequence of 24 conserved EPS genes showed divergence based on the associated the hosts (Figure 20B). This result indicated that to adapt to hosts, *L. salivarius* strains have developed ability to produce different EPS through point mutations rather than gene acquisition. This differs from the extracellular proteins that evolved by both HGT and mutation. It is suggested that the differences in substrates, sugars and peptides leads to the distinct evolution of EPS and extracellular proteins, although this is yet to be elucidated. This analysis of extracellular components offers evidence for and the direction of host adaptation of the human and chicken isolates that do not possess host-specific genes.

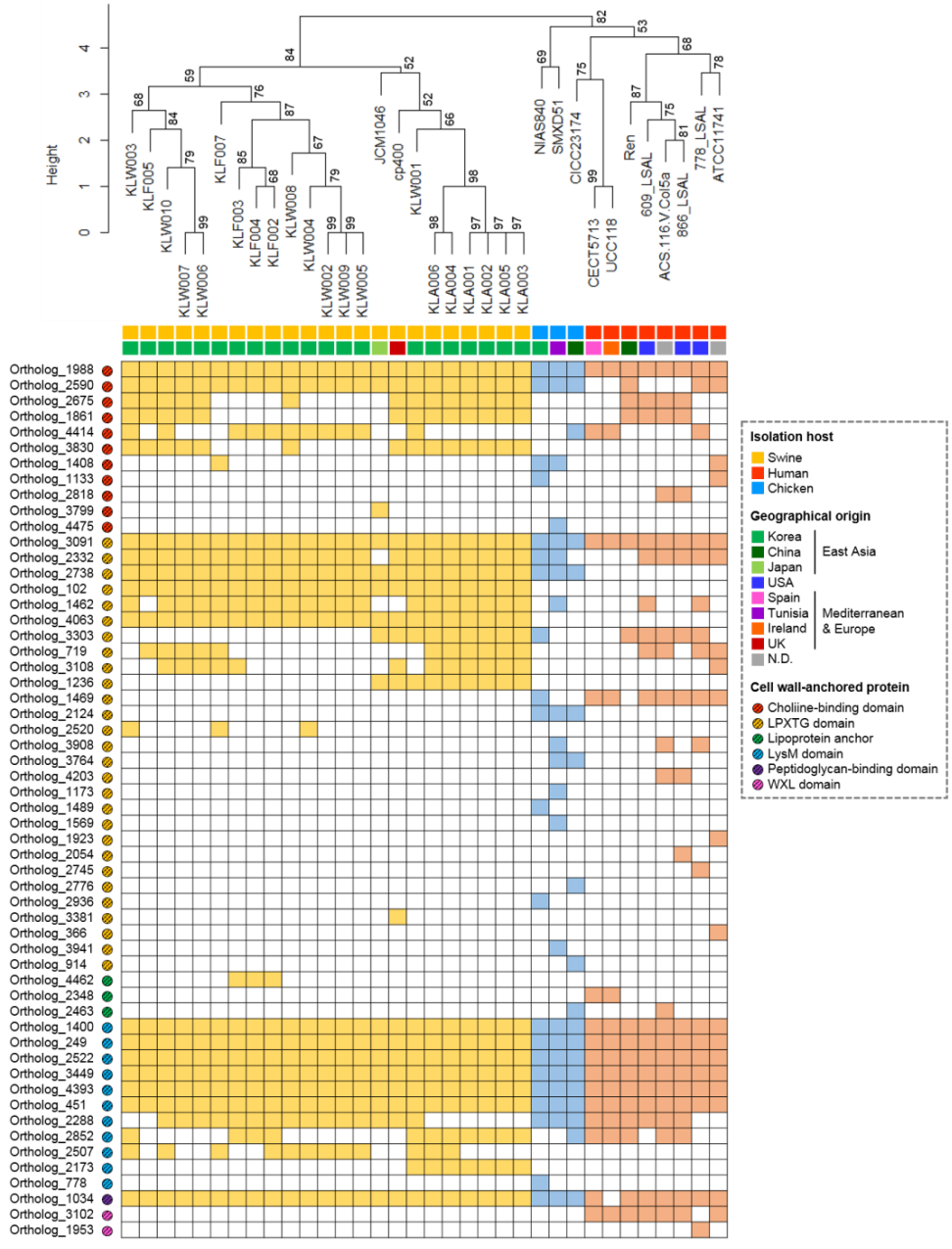


Figure 19. Phylogenetic clustering and distribution of extracellular protein encoding genes associated with adhesion property in *L. salivarius* genomes.

The colored box with black edge indicates gene presence in the genome. Yellow box, pig isolates; red box, human isolates; blue box, chicken isolates. A colored box with no edge indicates the isolation host and geographical origin of the bacterial strain. The colored circle with diagonal lines represents the type of cell wall-anchored domain. The color legends for the isolation host, geographical origin and the type of cell wall-anchored domain are given in the figure.

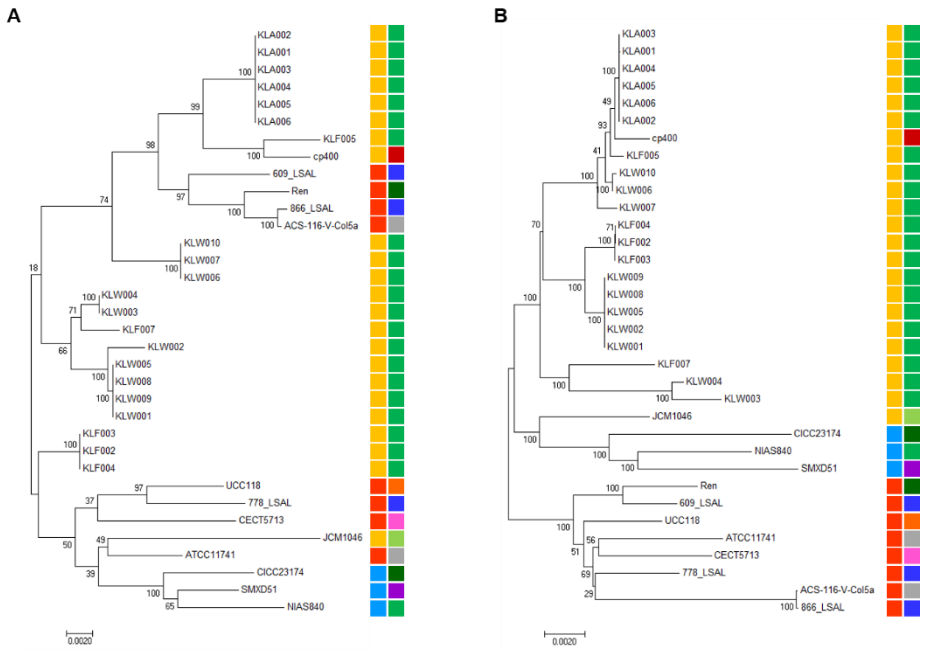


Figure 20. Phylogenetic clustering based on nucleotide sequence of conserved genes associated with extracellular components related to adhesion property.

Genes for eight extracellular proteins (A) and twenty-four EPSs (B) in *L. salivarius* pan-genome were used. The bootstrap consensus tree inferred from 1,000 replicates is taken.

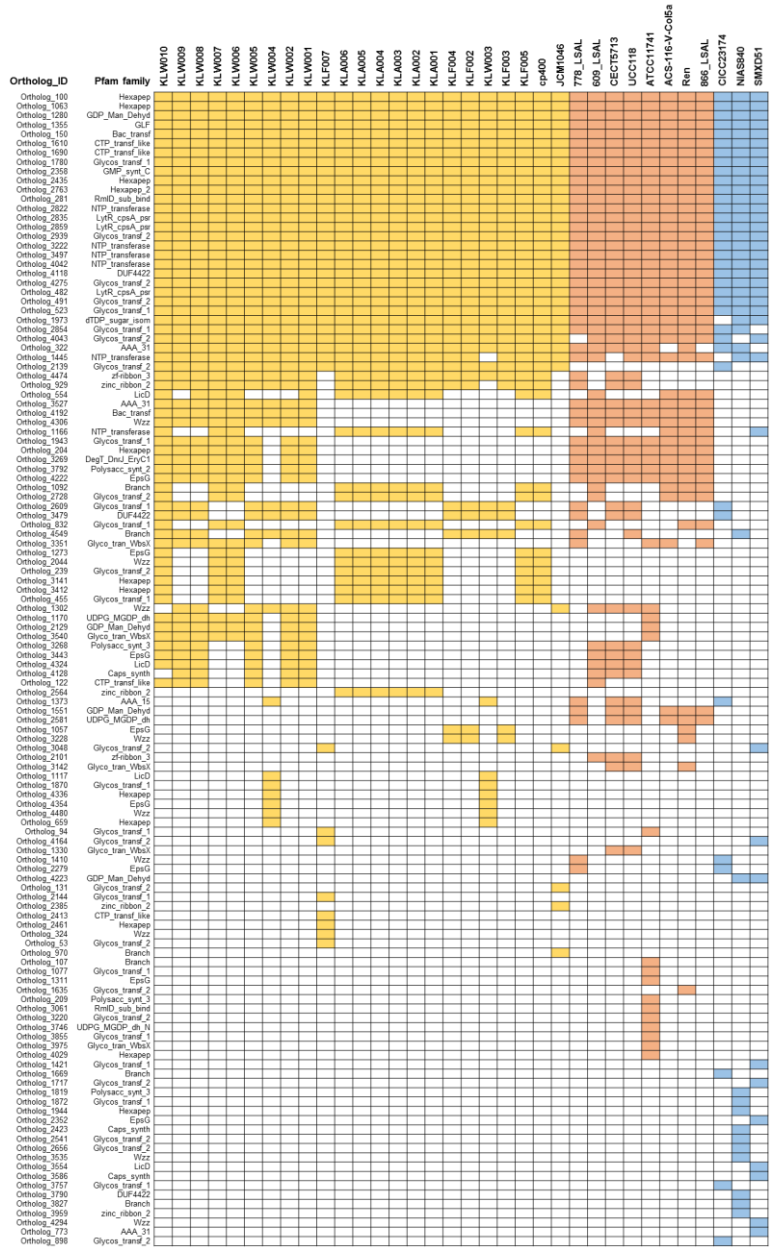


Figure 21. Presence of the genes related to EPS production associated with adhesion property of *L. salivarius*.

Colored box means gene presence in the genome. Yellow box, pig isolates; red box, human isolates; blue box, chicken isolates.

SecA2-SecY2 system

L. salivarius strains isolated from pigs and chickens have genes coding for the SecA2-SecY2 system, protein exportation machinery that the human *L. salivarius* isolates do not possess. This auxiliary protein secretion system is present in a limited number of Gram-positive bacteria and mycobacteria as well as other members of the Bacilli (Feltcher and Braunstein, 2012). The SecA2-SecY2 system facilitates the glycosylation of SRPs and exportation of the glycosylated SRP. Three pig isolate-specific genes and all genes shared by the pig and chicken strains are involved in this system (Figure 22). Pig and chicken isolates possess major genes for this protein secretion system, including *secA2*, *secY2*, three accessory secretory proteins genes (*asp1*, *asp2* and *asp3*), and several glycosylation genes, such as nucleotide sugar synthetase gene (*nss*) and glycosyltransferase gene (*gtf*). Among the 13 pig and chicken isolate-shared genes, one gene is unrelated to the SecA2-SecY2 system but is located in the locus of this system. Three pig isolate-specific genes, ortholog_43, ortholog_1434 and ortholog_2621, are also located at the SecA2-SecY2 loci. Ortholog_43 and ortholog_1434 are responsible for protein glycosylation as parts of the SecA2-SecY2 system.

It is believed that target protein of the SecA2-SecY2 system is a membrane-anchoring protein containing a serine-rich domain, and a gene encoding the target protein is located upstream of the *secY2* gene in the same reading direction (Feltcher and Braunstein, 2012). Furthermore, several studies revealed that SRPs

function in cell adhesion to the host surface (Siboo et al., 2005; Samen et al., 2007; Mistou et al., 2009). However, a gene with all the requirements for a target protein was not identified in the genomes of the pig and chicken isolates. Ortholog_2738 and ortholog_4332 have the most similar characteristics within the *L. salivarius* genomes (red arrow boxes in Figure 22). Like the SecA2-SecY2 system, ortholog_2738 is conserved in both pig and chicken isolates and encodes a surface protein SdrI containing a LPXTG motif and highly repeated serines (Table 6). However, ortholog_2738 is located between the genes for the SecA2-SecY2 machinery and glycosylation, and wide variation in gene size of the ortholog implies that this gene has been rapidly mutated and is therefore not important to survival. Ortholog_4332 encodes a secreted serine endoprotease that is the protein which is the most abundant in serine in the 10 kb upstream of the SecA2-SecY2 system. This enzyme is homologous with DegP, which is responsible for clearance of denatured proteins and stabilization of the cell membrane (Strauch and Beckwith, 1988; Strauch et al., 1989). Ortholog_4332 is conserved in all *L. salivarius* strains, although the human strains do not possess a SecA2-SecY2 system, implying that either the DegP homolog does not work in the human isolates or that ortholog_4332 is not a target protein of the SecA2-SecY2 system. Of note, genes for the SecA2-SecY2 system are highly conserved in the *L. salivarius* genomes, indicating that this protein exportation system has an important function for bacterial survival. It is suggested that the operational mechanism of this system in *L. salivarius* is different from other bacteria that possess a SecA2-SecY2 system, although more intensive study is needed.

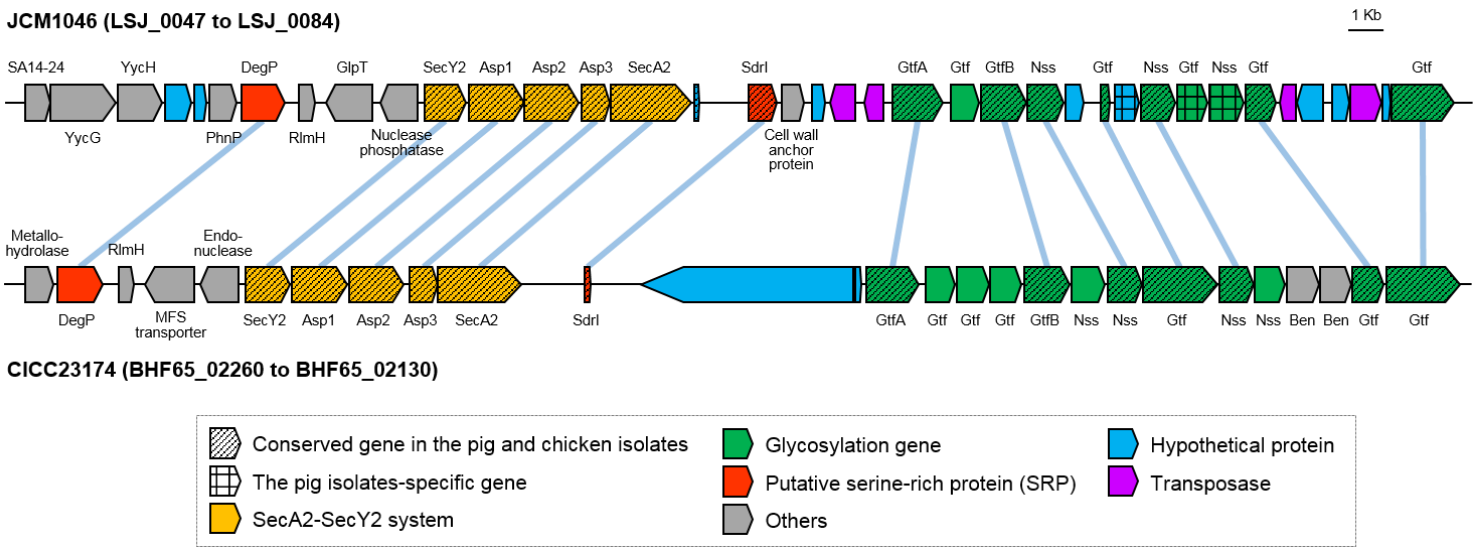


Figure 22. The SecA2-SecY2 system among the pig and chicken isolate-shared genes and their neighboring genes.

These genes are associated with adhesion property. Genomes of *L. salivarius* JCM1046 and CICC23174 were used for comparison. The azure line indicates the same orthologs. Abbreviations; SA14-24, two-component response regulator; YycG, two-component sensor kinase; YycH, regulator of two-component system; PhnP, Zn-dependent hydrolase of beta-lactamase superfamily; DegP, trypsin-like serine proteases; RlmH, 23S rRNA (pseudouridine(1915)-N(3))-methyltransferase; GlpT, glycerol-3-phosphate transporter; SdrI, serine-aspartate repeat-containing protein I; Gtf, glycosyltransferase; Nss, nucleotide sugar synthetase; Ben, benzoate transporter; CpsJ, capsular biosynthesis protein.

(2) Nutrient utilization

Among the identified host-specific genes, several genes are related to nutrient utilization. Commensal bacteria evolve in accordance with the nutritional conditions of the host environments (Hooper et al., 2002; Alverdy et al., 2005). Efficient nutrient usage is important for energy production in commensal bacteria, because higher energy consumption leads to the rapid growth of bacteria and helps competition with other microbes within the habitats. The differences in diet of the hosts generate differences in the intestinal environments that commensal bacteria colonize. *L. salivarius* acquired these host-specific genes associated with energy production to increase their fitness during evolutionary process.

Among the pig isolate-specific genes, ortholog_3862 is associated with energy metabolism. Ortholog_3862 encodes an argininosuccinate lyase that converts argininosuccinic acid to fumarate in the citric acid cycle (Figure 23A). This enzyme is involved in carbohydrate metabolism via the citric acid cycle, indicating that this lyase contributes to the energy production for the growth of the pig isolates.

In the *L. salivarius* strains isolated from chickens, ortholog_799 and ortholog_2329 are host-specific orthologs (Figure 23B). Ortholog_799 encodes a lactate 2-monooxygenase that converts lactate to acetate, and ortholog_2329 encodes a hexosephosphate transport protein (homologous with UhpT) that facilitates the cellular uptake of hexosephosphate. Ortholog_799 is located

alongside ortholog_2329 in the genomes of *L. salivarius* chicken isolates (BHF65_02290 and BHF65_02295 in CICC23174 genome, respectively), suggesting that these two genes are functionally associated. Glucose-6-phosphate is an important hexosephosphate for energy production. LAB convert glucose-6-phosphate to lactate during lactate fermentation, and secreted lactate is then converted to acetate by lactate 2-monooxygenase. Microbiome-derived SCFAs are considered as key players in regulating host obesity, and acetate is generally obesogenic in animals (Chakraborti, 2015). The administration of several lactobacilli, such as *L. fermentum*, *L. ingluviei*, *L. agilis* and *L. salivarius* was associated with weight gain in chickens (Lan et al., 2003; Khan et al., 2007; Angelakis and Raoult, 2010; Capcarova et al., 2010), while consuming some *Lactobacillus* species resulted in weight loss in other animals (Takemura et al., 2010; Karlsson et al., 2011; Sanchez et al., 2014). Taken together, it is postulated that lactate 2-monooxygenase and hexosephosphate transport protein-producing *L. salivarius* strains are helpful for both the weight gain of chickens and the growth of LAB. Because weight gain is one of the most important purposes to farm animals, chickens that have *L. salivarius* strains with increased lactate 2-moonooxygenase activity would be selected for during the improvement and breeding of chickens, especially broilers.

In this study, a human-specific *L. salivarius* gene involved in energy metabolism was not identified. Because humans are exposed to various environments that vary by region, such as foods, it is assumed that genes required

only in certain circumstances are not needed. In contrast, pigs and chickens are fed formulated feed with specified ingredients and are raised in farms, and therefore the commensal bacteria in the farm animals have fixed genomic contents for energy metabolism.

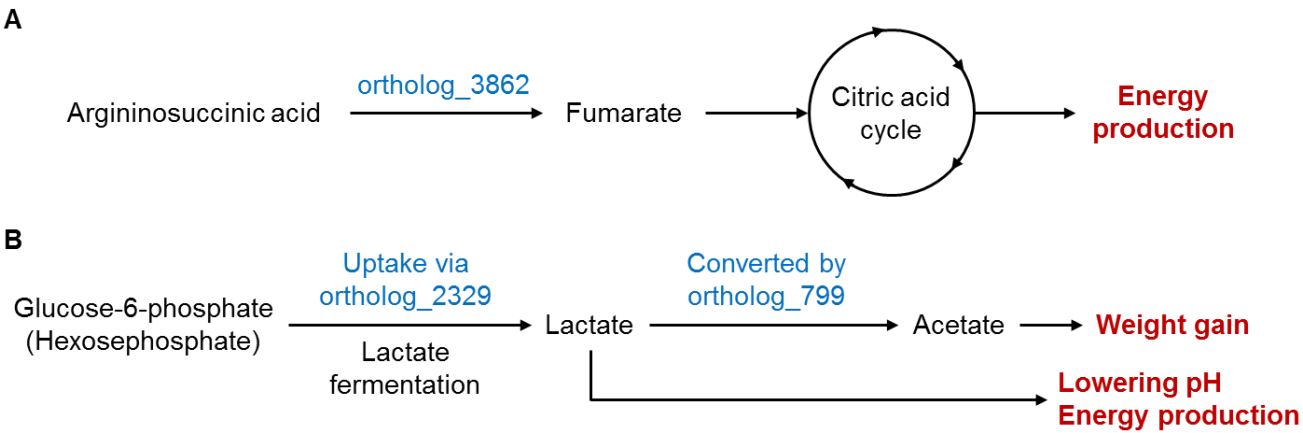


Figure 23. Host-specific genes associated with nutrient utilization of *L. salivarius*.

(A) The pig isolates-specific ortholog_3862 (argininosuccinate lyase). (B) The chicken isolates-specific ortholog_2329 and ortholog_799 (hexosephosphate transport protein and lactate 2-monooxygenase, respectively).

(3) Resistance against host stress

Ortholog_310 which is a pig isolate-specific ortholog encodes a choloylglycine hydrolase (bile salt hydrolase), which produces secondary bile salt in animal intestines. It had been reported that various *Lactobacillus* species have choloylglycine hydrolase activity (Bateup et al., 1995; Smet et al., 1995; Tanaka et al., 1999), and it is a desirable trait in probiotic bacteria. Liver cells of animal hosts synthesize primary bile acids, and secondary bile acids are converted by choloylglycine hydrolase. This enzyme has mutualistic functions that increase the fitness of both microbes and hosts. For microbes, choloylglycine hydrolase provides bile detoxification, gastrointestinal persistence and membrane alterations, and for hosts it alters digestive functions and lowers cholesterol (Begley et al., 2006). The *L. salivarius* pan-genome possesses three choloylglycine hydrolase genes, including ortholog_310, and all the *L. salivarius* strains possess more than one choloylglycine hydrolase ortholog (Figure 24). This observation agrees with the results of a previous study that showed other *Lactobacillus* species also have multiple genes for this hydrolase (Begley et al., 2006).

Interestingly, while human and chicken isolates possess one or two choloylglycine hydrolase genes, most pig isolates (22 of 23 strains) have three genes (including ortholog_310). It is suggested that the pig intestine requires more secondary bile salts than other host habitats, because the environment where the pig isolates inhabit may be not favorable. When considering that domestic pigs are selected and bred to increase weight gain and fat accumulation, they would

have been selected with production ability of more bile acid, which contributes lipid absorption. Indeed, pigs secrete more bile than humans, although the quantity is dependent on what they eat. Pigs secrete 1200 to 1700 ml of bile per day, but 600 to 800 ml per day in humans (Sambrook, 1981; Bowen, 2001; Boyer, 2013). Because more bile secretion in the pig intestine is highly toxic to the intestinal bacteria, the bacteria would need more choloylglycine hydrolase to protect them from bile. However, another possibility cannot be excluded that ortholog_310 is genetically linked with ortholog_102, which encodes a pig isolate-specific MucBP. Indeed, four pig isolate-specific orthologs (ortholog_102, ortholog_310, ortholog_3201 and ortholog_3862) are located close to each other in the genome of the pig isolates (Figure 25), suggesting that these four orthologs might have been integrated together into the genomes of the pig isolates by HGT.

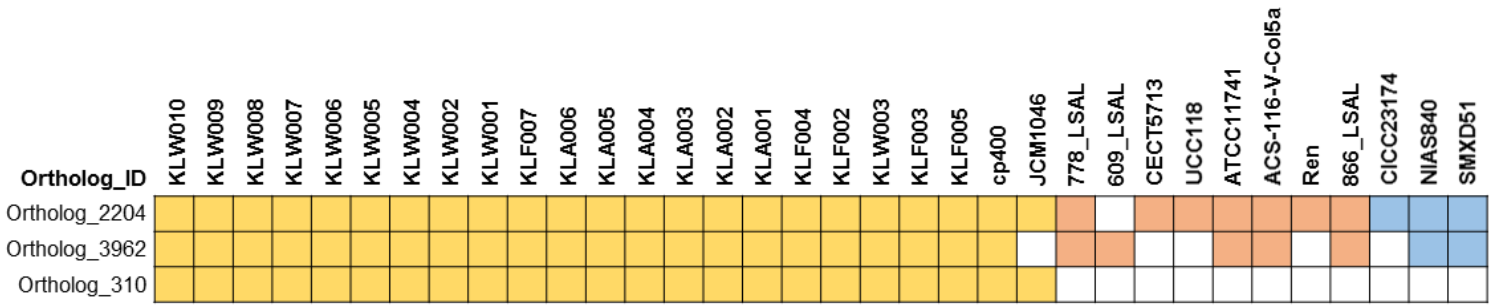


Figure 24. Presence of the *L. salivarius* choloylglycine hydrolase genes associated with ability for resistance against host stress.

Colored box means gene presence in the genome. Yellow box, pig isolates; red box, human isolates; blue box, chicken isolates.

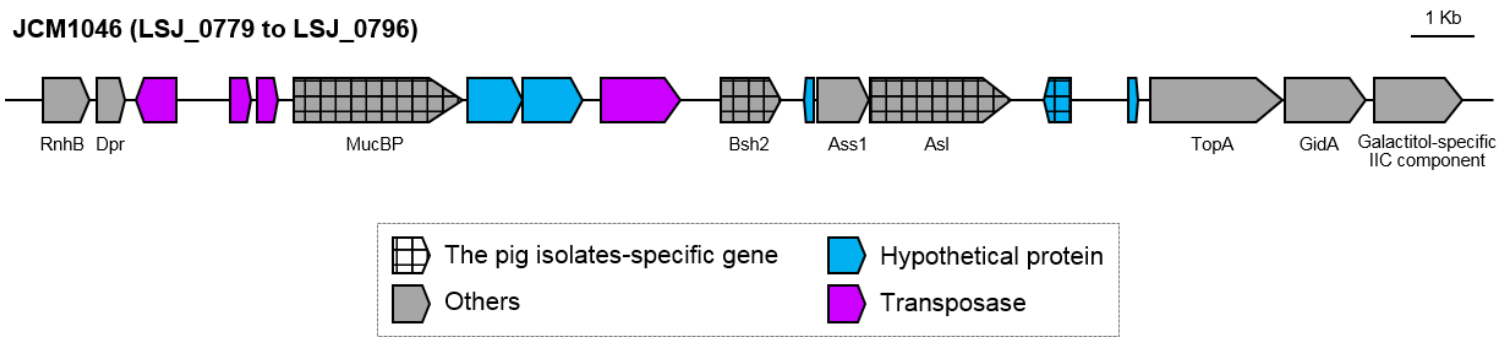


Figure 25. Pig isolate-specific genes and their neighboring genes.

A genome of *L. salivarius* JCM1046 was used. The azure line indicates the same orthologs. Abbreviations; RnhB, ribonuclease HII; Dpr, DNA processing protein; MucBP, mucus-binding protein; Bsh2, choloylglycine hydrolase (bile salt hydrolase 2); Ass1, argininosuccinate synthase; Asl, argininosuccinate lyase; TopA, DNA topoisomerase I; GidA, glucose-inhibited division protein A.

(4) Other host-specific or dual-host-shared genes

Ortholog_2124 is a chicken isolate-specific gene and is homologous with the *sea1*/*prgA*, which encodes a surface exclusion protein. This gene is a located on the pTEF2 plasmid, which originated from *E. faecalis* (Manson et al., 2010). The pan-genome of *L. salivarius* possesses six surface exclusion protein genes, none of which is conserved among the *L. salivarius* strains (Figure 26). This gene is not identified as being important in the chicken strains, but it is assumed that an ancestor of the chicken isolates had been conjugated with an F^+ *E. faecalis* and obtained the ortholog_2124 gene (or the entire pTEF2 plasmid).

The pig and human isolates shared two genes, ortholog_401 and ortholog_3356, encoding exodeoxyribonuclease VII small subunit and a hypothetical protein, respectively. The exodeoxyribonuclease VII encoded by ortholog_401 (homologous with *xseA*) is an enzyme that catalyzes the exonucleolytic cleavage of DNA. Although this nuclease prefers single-stranded DNA and mediates mismatch repair in *E. coli* (Burdett et al., 2001), it is not known if it also provides the same function in lactobacilli. This enzyme is composed of large and small subunits, and the large subunit gene (ortholog_3308, homologous with *xseB*) is conserved in all *L. salivarius* strains. A gene for the small subunit is absent in the chicken isolates, indicating that there is no exodeoxyribonuclease VII activity in the chicken strains, although further study is needed to determine why small subunit gene of this enzyme is lost in the *L. salivarius* strains isolated from chickens.

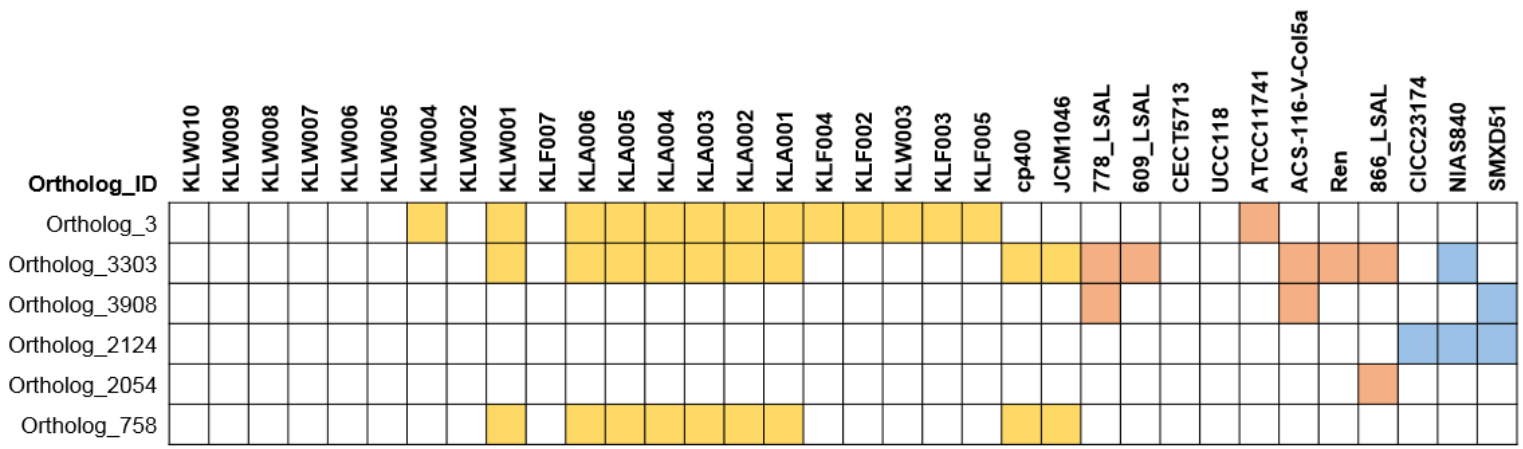


Figure 26. Presence of the surface exclusion protein genes in the *L. salivarius* genomes.

Colored box means gene presence in the genome. Yellow box, pig isolates; red box, human isolates; blue box, chicken isolates.

(5) Antibiotic resistance genes

Bacteria that colonize farm animals have acquired AR genes for their survival in an environment in which farmers use AGPs to increase the performance and/or productivity of the animals. AGPs have provided increased productivity for farmers, and the agricultural industries and the consumers have benefited from AGPs. The presence of AR genes in the *L. salivarius* genomes was confirmed using the CARD database (Figure 27). The human strains did not possess AR genes, although most pig and chicken strains had more than two AR genes (21 of 23 pig isolates and 2 of 3 chicken isolates). This result is related to the exposure frequency of the hosts to antibiotics. Farm animals have fed the feeds mixed with AGPs to increase their productivity, and therefore they were constantly exposed to antibiotics over a long time. Thus, the *L. salivarius* isolates that originated from pigs and chickens might have acquired the AR genes for their survival. In the case of humans, antibiotics were not abused and used only for clinical purposes. This difference in the use of antibiotics explains the different distribution of AR genes of commensal bacteria of different hosts.

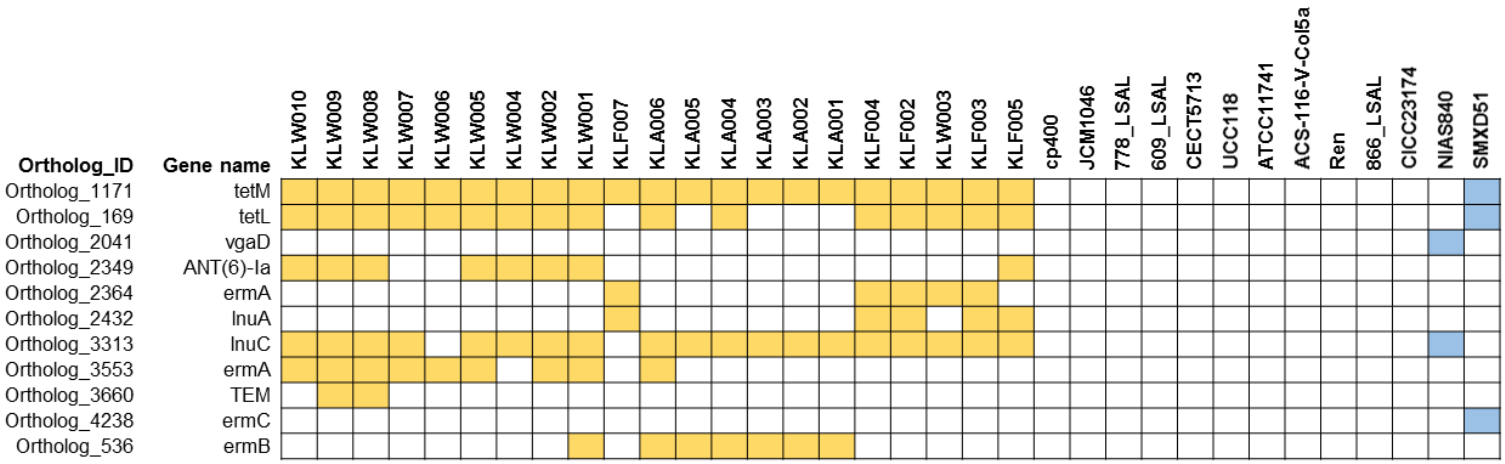


Figure 27. Presence of the genes for AR genes in the *L. salivarius* genomes.

Colored box means gene presence in the genome. Yellow box, pig isolates; blue box, chicken isolates. All human isolates (from 778_LSAL to 866_LSAL, eight strains) did not possess AR genes.

3) *L. salivarius* strains isolated in this study

(1) Genomic features of the *L. salivarius* isolated in this study

As described previously, *L. salivarius* strains at an interval of a decade were isolated and the use of AGPs was legally banned in Republic of Korea during this period. Six SBPs and fifteen SAPs were isolated from the two time points, before and after the ban on AGPs, respectively. The ban on using AGPs changed the conditions of the livestock industry, including the physiological characteristics of the farm animals and their commensal bacteria. It is well documented that alpha diversity of the intestinal microbiota was decreased following antibiotic perturbation in animals (Antonopoulos et al., 2009; Greenwood et al., 2014; Panda et al., 2014).

Focusing only on the orthologs of the genomes obtained in this study, they had 3,318 total orthologs, including 1,384 core genes and 322 stain-specific orthologs (Figure 28). The number of orthologs only appeared in SBPs (335 orthologs) and was higher than the number of SAP-specific orthologs (103 orthologs). As shown in Table 4, the average genome size of SBPs (2.28 ± 0.04 Mbp) was significantly larger than that of SAPs (2.18 ± 0.13 Mbp, P = 0.01), and SBPs had a higher number of CDSs (2189.50 ± 37.2 of CDSs) than SAPs (2054.40 ± 118.81 of CDSs, P = 0.001). GC% was not different between the two groups (SBPs = 32.84 ± 0.04%, and SAPs = 32.81 ± 0.10%).

To confirm whether SBPs and SAPs are different in the genomic contents,

hierarchical clustering of the genomes was carried out. The clustering based on 3,318 orthologous CDSs showed that the 6 SBPs were distinguished from 15 SAPs (Figure 29A). Another phylogenetic analysis based on the sequences of seven MLSA genes also showed that SBPs are strictly separated from SAPs (Figure 29B). Considering these distinctions between the two groups, it is evident that SBPs formed an indisputably distinct lineage from SAPs, and AGPs in feeds affect the genomic contents of *L. salivarius* that colonized the porcine intestine. Furthermore, the genetic distance between SBPs was closer than SAPs and this indicated that SBPs had more similar genomic features, although SBPs were isolated from more variable swine farms than SAPs.

The genomic features of SBPs, including the number and the contents of orthologs, were barely different among the six SBPs. However, the genomic profiles of SAPs were more diverse than SBPs. This result may explain two evolutionary points: 1) AGPs in the feeds had caused the unremitting and strong selective antibiotic challenge to intestinal bacteria; 2) After the prohibition of AGPs, intestinal bacteria had been released from the antibiotic selection pressure, and this situation allows more evolutionary freedom. The ban of AGPs forced ancestral strains of SAPs to gradually change their genomic contents through the movement of genetic material, such as HGT. During the evolutionary process, ancestral strains of SAPs would have discarded several genes that were not needed for the survival of the bacteria, leading to the smaller genome size and decreased number of CDSs of SAPs compared to SBPs.

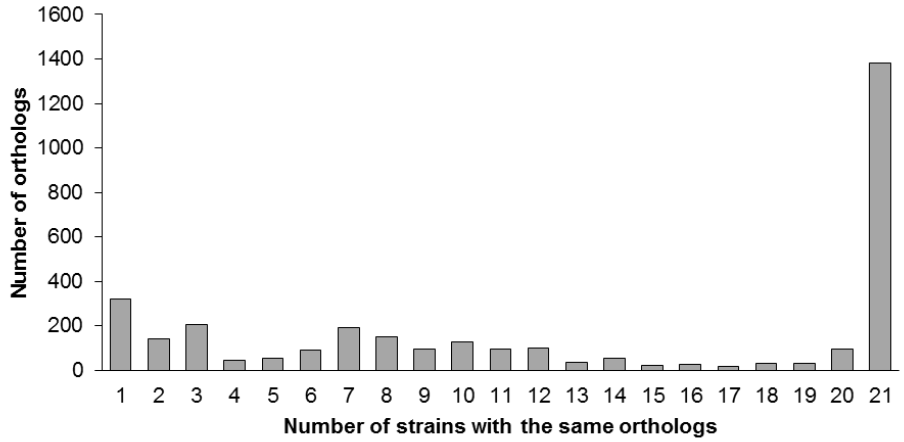


Figure 28. Distribution of orthologous CDS in 21 *L. salivarius* isolated in this study.

Distribution histograms are shown for 21 *L. salivarius* isolated in this study. The horizontal axis indicates the number of isolates sharing the same orthologous CDS, and vertical axis represents the number of orthologous CDS shared by the indicated number of isolates.

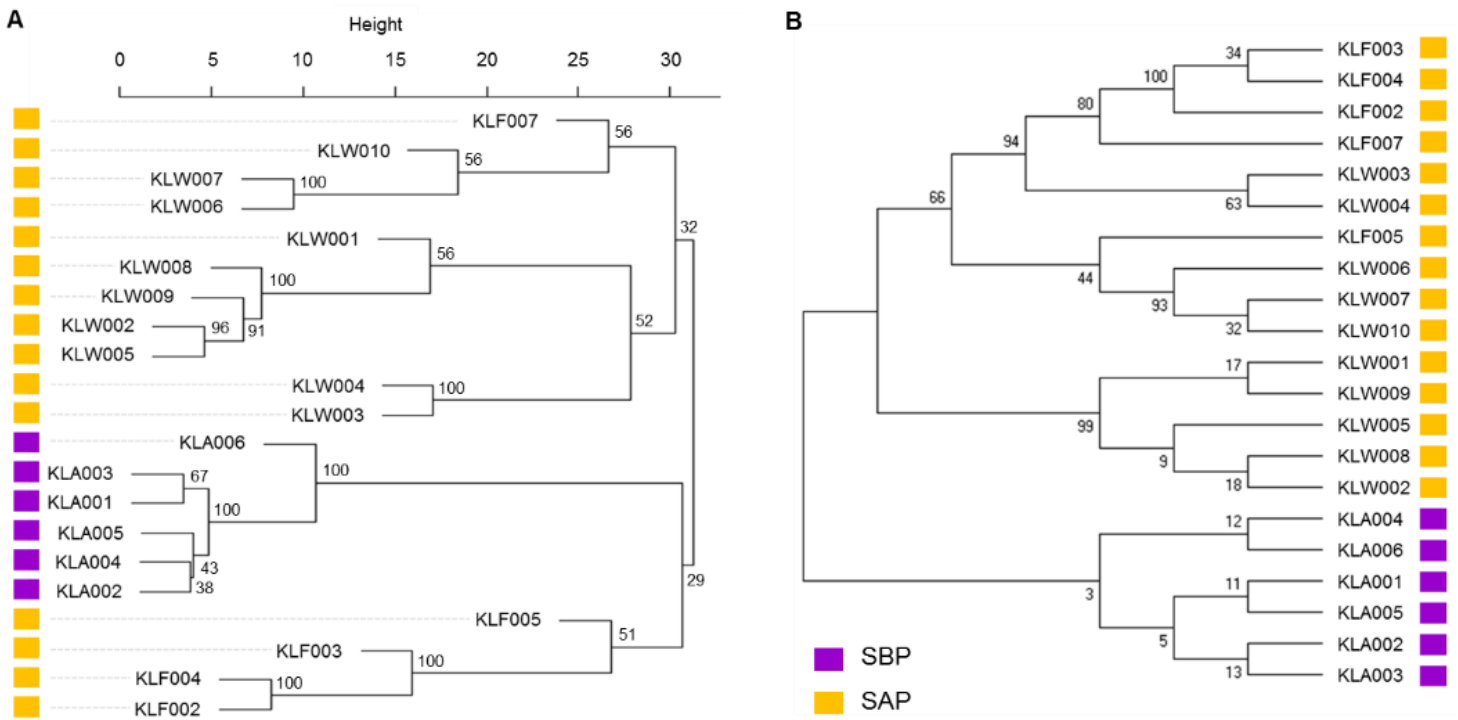


Figure 29. Phylogeny of the isolated *L. salivarius* strains.

(A) Hierarchical clustering of *L. salivarius* strains based on orthologous CDS contents. (B) Phylogeny of MLSA sequences found in *L. salivarius* strains.

(2) Antibiotic resistance of SAPs and SBPs

Bacteria that had colonized the animal intestines had acquired AR genes for their adaptation to the environment in which farmers had used AGPs. The presence of AR genes in the genomes was determined using a database of AR genes, the CARD (Figure 30). All of the strains had three to five AR genes and there was no difference in the number of AR genes existing in the genomes, although it is no longer necessary for SAPs to maintain the AR genes after the use of AGPs was banned. The ancestors of SAPs acquired AR genes to protect themselves against antibiotics when AGPs were used freely, and SAPs have maintained these AR genes inherited from their ancestors until recently.

The different distribution of AR genes in SBPs and SAPs might be influenced by isolated farms, but these genes were analogous in their functions. The AR genes target the same antibiotics class, protein synthesis inhibitors, except the *blaTEM* gene, which protects bacteria from penicillins, one of the cell envelope antibiotics, indicating that functionally similar or identical AGPs had been used in the swine farms of Republic of Korea. Most of these AR genes are act by blocking the protein synthesis inhibitors to protect *L. salivarius* strains from lincosamides (*ermA, ermB, lnuA, lnuC* and *vatE*) and tetracyclines (*tetC, tetL* and *tetM*). Other two AR genes, *ant(6)-Ia* and *fexA*, have a defensive function against aminoglycosides and amphenicols, respectively. All of these antibiotics have been used as AGPs in swine farms of Far-East Asia (Zhou et al., 2013; Zhu et al., 2013), and genes that target the antibiotics described above were found in other bacterial

species isolated from the feces of pigs and cattle in Republic of Korea (Lim et al., 2007).

Swine intestinal microbes that did not have AR genes could not protect themselves from AGPs when antibiotics were added to animal feeds. Furthermore, several studies showed that intake of antibiotics decreased the diversity of microbiota in the animal intestines (Antonopoulos et al., 2009; Greenwood et al., 2014), although the total number of intestinal bacteria was not changed (Panda et al., 2014). In these circumstances, the competition between microbes for survival and domination in the porcine intestines would be decreased, and bacteria that had AR genes are more advantaged for survival in this microenvironment. On the other hand, increased competition after AGP prohibition required another strategy for survival among bacteria. They had to evolve for overcoming this severe struggle, and they should be improved their ability contributing survival in the habitats.

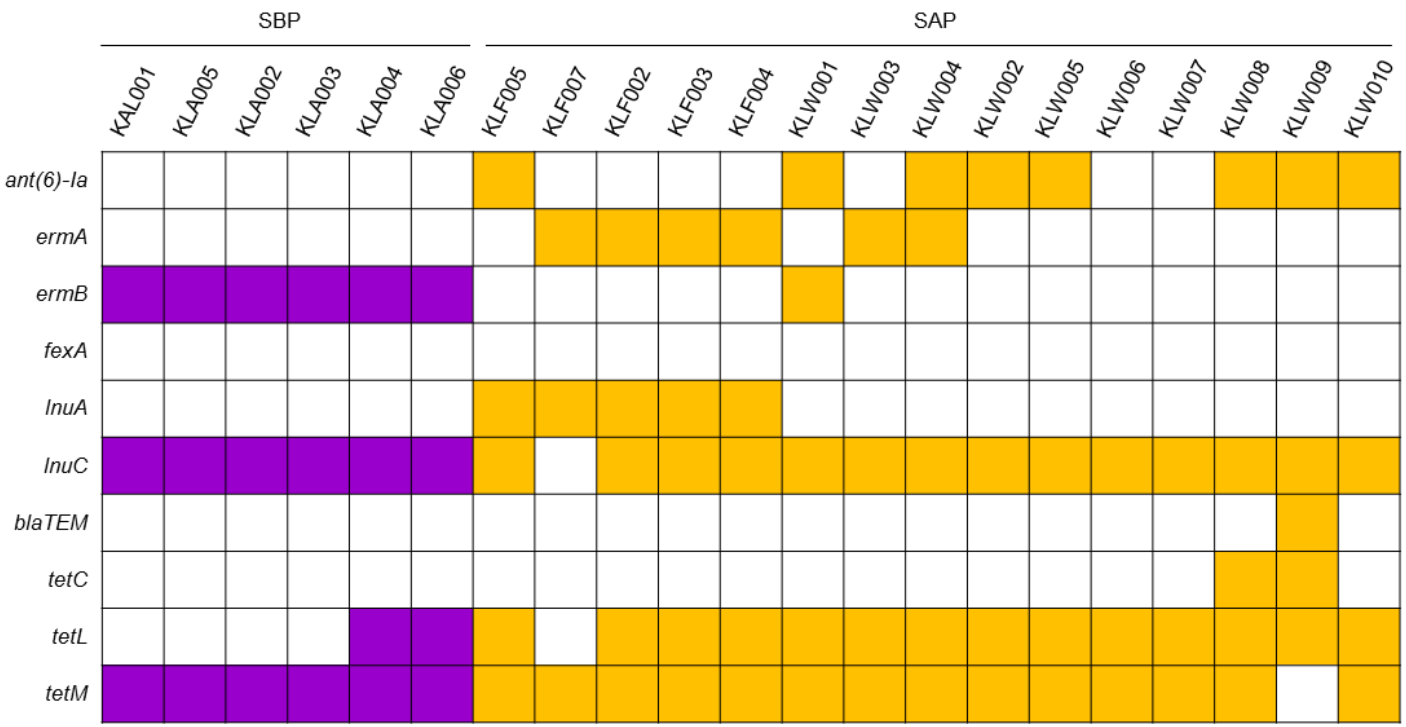


Figure 30. Comparison of AR genes of the 21 isolated strains.

Nucleotide sequences of AR genes were obtained from CARD. Orthologs of *L. salivarius* strains were aligned to the database using BLAST+, and orthologs showed E-value = 0, identity ≥ 90% were regarded as AR genes (colored square).

(3) Functional differences in genomic contents

Genomic annotation using the RAST server was carried out to identify genomic differences affecting bacterial survival ability between SAPs and SBPs. 10 RAST subsystems that are significantly different between the groups were found ($P < 0.05$, Figure 31). Among these subsystems, L-rhamnose utilization and D-tagatose and galactitol utilization of SBPs had over seven genes per strain for each subsystem, and these genes were almost absent in the genome of SAPs. These two subsystems are responsible for energy metabolism; Genes for L-rhamnose utilization were incorporated in glycolysis and pyruvate metabolism (Fructose and mannose metabolism, KEGG pathway ec00051), and genes for D-tagatose and galactitol utilization were related to the pentose phosphate pathway (Galactose metabolism, KEGG pathway ec00052). It is documented that L-rhamnose is utilized as a carbon and energy source by various microorganisms such as *P. stipitis, E. coli, B. cenocepacia, C. phytofermentans, S. spinose*, *S.* Typhimurium, and *A. pyridinolis* (Levinson and Krulwich, 1976; Obradors et al., 1988; Tobin and Schleif, 1990; Madduri et al., 2001; Cardona et al., 2006; Koivistoinen et al., 2012; Petit et al., 2013), and *L. salivarius* UCC118 is also capable to use L-rhamnose to produce energy via pentose phosphate pathway (Lebeer et al., 2008). Although there is no study that *L. salivarius* uses galactitol and D-tagatose for energy production, other lactobacilli utilize these sugars as energy sources; *L. plantarum* and *L. paracasei* use galactitol (Smokvina et al., 2013; Wang et al., 2016), and *L. casei, L. plantarum* and *L. sakei* use D-tagatose (Chouayekh et al.,

2007; Rhimi et al., 2010; Kang et al., 2013). D-tagatose and galactitol are also involved in the galactose metabolic pathway which is conserved in bacteria, indicating that a subsystem for D-tagatose and Galactitol Utilization help to produce energy for bacterial life. These reports imply that SBPs having the subsystems for utilization of L-rhamnose, D-tagatose and galactitol are capable enough to use these sugars as energy sources.

Furthermore, these carbohydrates can be used as a sugar backbone of a nucleotide. As described above, L-rhamnose is a source of the pentose phosphate pathway, which is capable to produce energy and ribose-5-phosphate for nucleotide synthesis (Mayes and Bender, 2003). The products from the galactose metabolism are also used for both energy production and nucleotide synthesis (Kilstrup et al., 2005). It is implied that the excessive nucleotide synthesis using these sugars would contribute to resist against some antibiotics. The mode of action of antibiotics is distinguished to three categories as described it above (Figure 2). Among the antibiotics, nucleoside (or nucleotide) analogues are antibiotics that are incorporated into growing DNA during DNA replication and/or disturb peptidoglycan layer of bacterial cell wall (Périgaud et al., 1992). Other antibiotics, such as sulfonamide and trimethoprim, prevent synthesis of tetrahydrofolic acid which is an important source of nucleotide production (Brown, 1962; Hitchings, 1973). When considering the condition that AGPs are prevalent in livestock farms, bacteria inhabiting to the farm animals including SBPs would had tried to increase their fitness against antibiotic threats. It is assumed that the

bacteria had secured two defense strategies against antibiotics; 1) acquisition of AR genes and blocking antibiotics directly, 2) utilization (or gain) of a salvage pathway to avoid antibiotic effects, such as use of L-rhamnose, D-tagatose, and galactitol for nucleotide synthesis. After the ban of AGPs, SAPs lost the subsystems for utilization of L-rhamnose, D-tagatose and galactitol, indicating that the need to produce more energy and nucleotide using these subsystems has diminished. The other eight subsystems showed significant differences between groups, but these subsystems sparsely existed in the 21 strains.

The presence of genes participating to the metabolic pathways of energy production and nucleotide synthesis would affect the sparse biofilm formation of SBPs. Compared to SAPs, SBPs have additional genes for energy production and nucleotide synthesis, including L-rhamnose utilization and D-tagatose and galactitol metabolism. L-rhamnose is the main structural component of EPSs produced in *Lactobacillus* spp. (Kojic et al., 1992; Tallon et al., 2003), and D-tagatose also contributes to EPS synthesis and lactic acid fermentation for energy production in LAB (Harutoshi, 2013). It is possible that SBPs use L-rhamnose as an energy or nucleotide source rather than for EPS production, and the genes for D-tagatose utilization are also associated with this energy production process. Due to the additional genes for sugar utilization, SBPs have more available energy than SAPs, and this energy would be used for other biological processes, such as choline and betaine uptake and betaine biosynthesis, and DNA replication strays. Moreover, SBPs showed a broader genetic capacity than SAPs, such as genome

size and number of orthologs, and 335 unique orthologs appeared in the SBP genomes, although SAPs has 103 unique orthologs. These differences in the genetic profiles suggest that the extra energy of SBPs would be used for other processes and excessive nucleotide synthesis of SBPs would help to increase their survivality against antibiotics rather than EPS production.

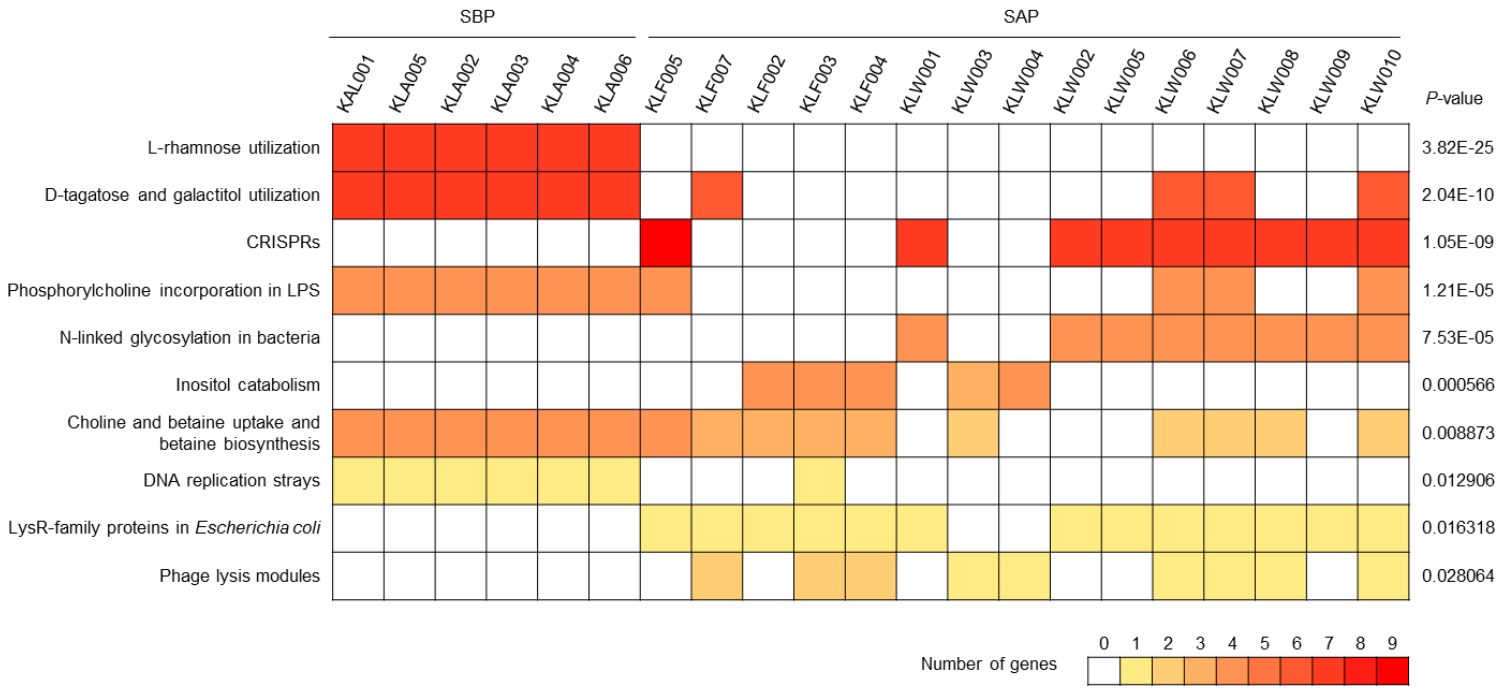


Figure 31. Comparison of functional features of the isolated *L. salivarius* genomes.

The genomes were annotated and grouped by RAST with default options for bacteria. The different colors showed the number of genes that incorporated in each subsystem (see color bar). Statistical analysis was carried out using Fisher's exact test between SBPs and SAPs, and the significantly different subsystems were shown in the Figure ($P < 0.05$).

(4) Genes related to EPS and cell wall proteins in SBPs and SAPs

L-rhamnose and D-tagatose are not only incorporated in the energy production and nucleotide synthesis pathways, but are also important for EPS production (Kojic et al., 1992; Tallon et al., 2003; Harutoshi, 2013). It is postulated that SBPs would rather use L-rhamnose and D-tagatose as sources for production of energy and nucleotide than for EPS production, due to the presence of the genes for utilization of these sugars considering their associated metabolic mechanisms. If SBPs used these sugars for energy and nucleotide production rather than EPS synthesis, their genomes would have changed to reflect the decreased use of these sugars for EPS synthesis.

Therefore, the absence or presence of the genes associated with EPS production was investigated. As described above, 217 genes related to EPS synthesis were collected by screening against Pfam, including 50 genes used in the previous study (Raftis et al., 2011). Among them, 128 orthologs including 40 core genes were found in the SBPs and SAPs. Gene presence in SBPs was different from the genomes of SAPs, furthermore, the phylogenetic diversity of the amino acid sequence of the genes with hierarchical clustering also revealed the distance between SBPs and SAPs (Figure 32). In Gram-positive bacteria, EPSs are important membrane components for niche adaptation and colonization through the biofilm formation (Vélez et al., 2007). It is suggested that the bacteria improved their ability for biofilm formation by the mutation and gene acquisition in EPS genes.

The genetic profiles for the extracellular proteins which are also associated to biofilm formation were also determined. Among the 56 extracellular protein-encoding orthologs in the pan-genome, 30 orthologs were observed in the SBPs and SAPs, consisting with 7 choline-binding domains, 11 LPXTG domains, one lipoprotein anchor, 10 LysM domains and one peptidoglycan-binding domain. Phylogenetic clustering showed that the ortholog distribution of SBPs was distinct from SAPs, implying the evolutionary differences between the groups (Figure 33). Furthermore, hierarchical clustering based on amino acid sequences of the 14 core genes for extracellular proteins in the SBPs and SAPs also showed the genetic distance between them (Figure 34).

The analyses of EPS and extracellular protein genes revealed that the nucleotide sequence of the SBP genes was distinct from SAPs, suggesting that these differences would affect the biofilm formation. Indeed, EPS and extracellular protein are important components of cell wall and biofilm in lactobacilli (Lebeer et al., 2008; Raftis et al., 2011). Biofilm provides the sufficient power to oppose other microbes (Nadell et al., 2009; Hibbing et al., 2010). Bacteria isolated from the time when AGPs were added in the feeds did not need to form biofilm because AGPs decreased the competition among microbes. This suggests that the differences in these genes would have a relationship with the amount or characters of biofilm produced by SBPs and SAPs, and dense biofilm formation leads to increased survivability of SAPs from competition with intestinal microbes.

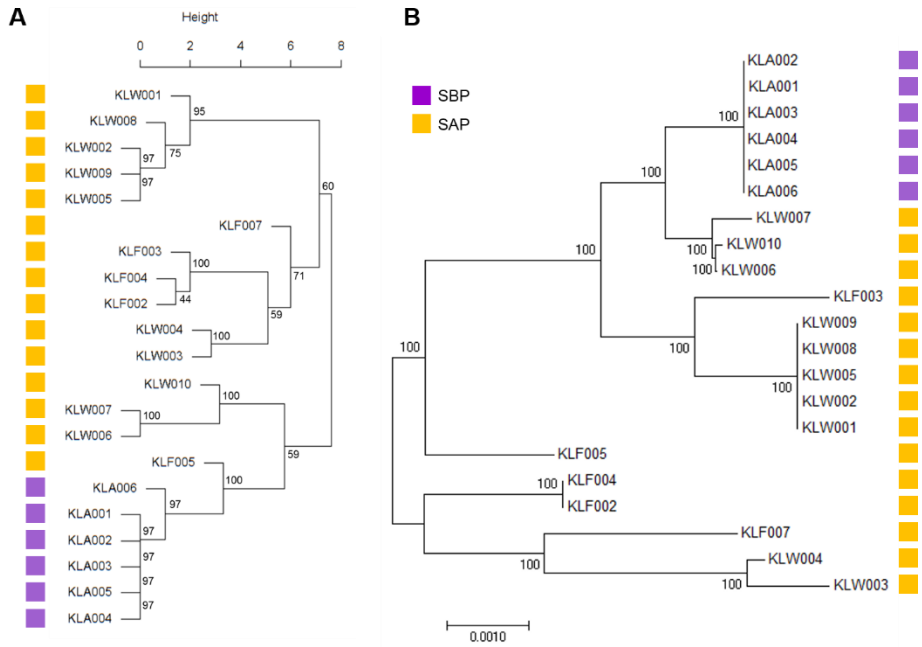


Figure 32. Phylogenetic clustering with EPS-related genes of SAPs and SBPs.

(A) Tree based on the presence of 128 EPS-related genes of SAPs and SBPs. (B) Hierarchical clustering based on amino acid sequences of the 40 core genes for EPS production. The bootstrap consensus tree inferred from 1,000 replicates is taken.

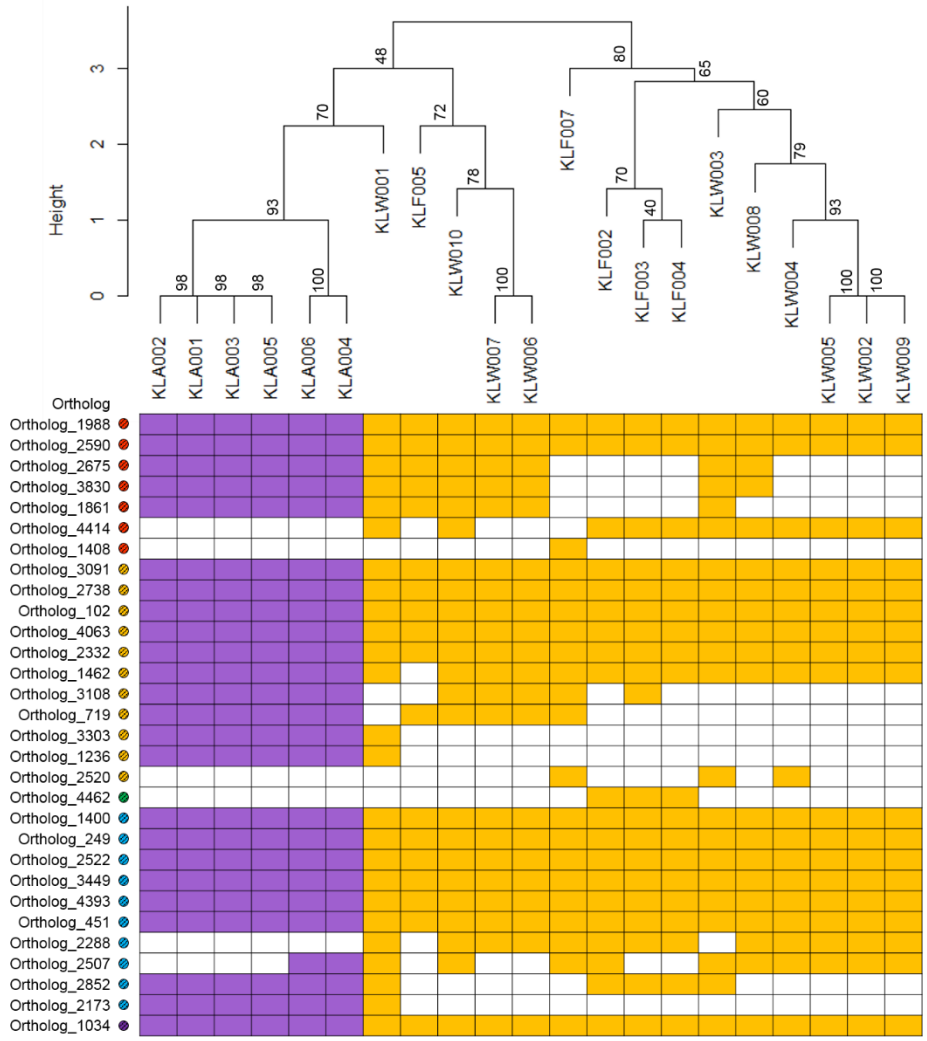


Figure 33. The presence of extracellular protein genes in the isolated *L. salivarius* strains.

Colored box with black edge means gene presence in the genome. Purple box, SBPs; gold box, SAPs. Circles with diagonal line mean types of cell wall-anchored proteins. Red, choline-binding domain; yellow, LPXTG domain; green, lipoprotein anchor; blue, LysM domain; purple, peptidoglycan-binding domain. The bootstrap consensus tree inferred from 1,000 replicates is taken.

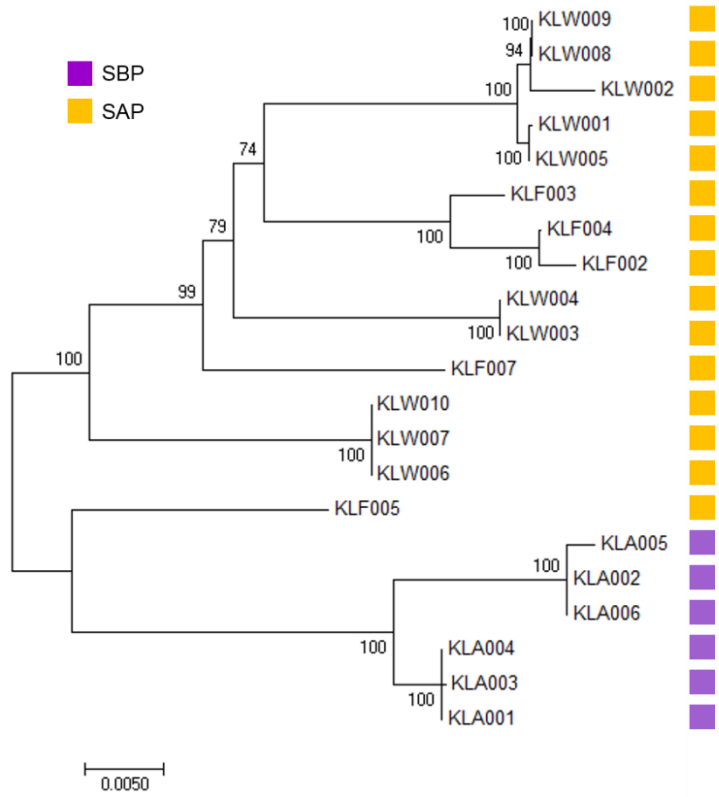


Figure 34. Hierarchical clustering based on amino acid sequences of the 14 core genes for extracellular proteins in the isolated 21 *L. salivarius* strains.

The bootstrap consensus tree inferred from 1,000 replicates is taken.

(5) *In vitro* biofilm formation and competition with intestinal microorganisms

EPSs and extracellular proteins consist Gram-positive bacterial cell wall and are important for biofilm formation of *L. salivarius* (Lebeer et al., 2008; Raftis et al., 2011). Furthermore, different cell aggregation between SBPs and SAPs was observed (Figure 35). Cell aggregation is also affected by cell wall components and associated with biofilm formation (Costerton et al., 1995). Considering that the distribution and peptide sequences of EPS and extracellular protein genes were different between SBPs and SAPs, it is assumed that ability for biofilm formation of the strains would be different. *In vitro* biofilm formation of the *L. salivarius* strains was tested, and significant differences were observed between the groups (Figure 36A). SAPs formed more biofilm than SBPs after 48 and 72 h of cultivation. SAPs formed 11-fold more biofilm in 48 h and 30-fold in 72 h than SBPs. Quantification of dye bound to biofilm for SBPs showed 3.79 ± 1.62 μg in 48 h and 2.05 ± 0.57 μg in 72 h of, while SAPs showed 40.25 ± 4.64 μg in 48 h and 62.38 ± 4.72 μg in 72 h.

The strains that form more biofilm than others have a survival advantage on the competing species and can become a dominant bacterium in certain habitat, such as the porcine intestine. The survival rate of SAPs in competition with swine intestinal microbes was corroborated (Figure 36B). The number of SAP cells increased after 48 h compared to 24 h, while the SBP cells had decreased. The number of *L. salivarius* cells in co-cultures of SAPs and intestinal microorganisms

for 24 h did not differ from SBPs. However, after 48 h of competition, the number of SAP cells was higher than SBPs ($P = 8.92 \times 10^{-10}$). These results indicate that SAPs were suitable to survive because of their ability to form biofilm.

The ability for biofilm formation that helps to survive in the competition with intestinal microorganisms is an important probiotic property. *L. salivarius* has many features as probiotic, including antimicrobial activity against pathogenic bacteria (Flynn et al., 2002; Corr et al., 2007), reduction of pathogen adhesion to surfaces (O'Hara et al., 2006), bile salt hydrolase activity (Fang et al., 2009), indirect support of energy metabolisms by microbial fermentation (Maldonado et al., 2010) and effects on host immunocompetent cells (O'Mahony et al., 2006). To maximize these probiotic functions, *L. salivarius* have to colonize and survive in the GIT, and biofilm formation helps this process. This spatial organization of microbes attached to the intestinal surfaces subdivides the role of the members and protects themselves from harmful substances and other competitive microorganisms (Hooper and Gordon, 2001; Nadell et al., 2008; Hibbing et al., 2010).

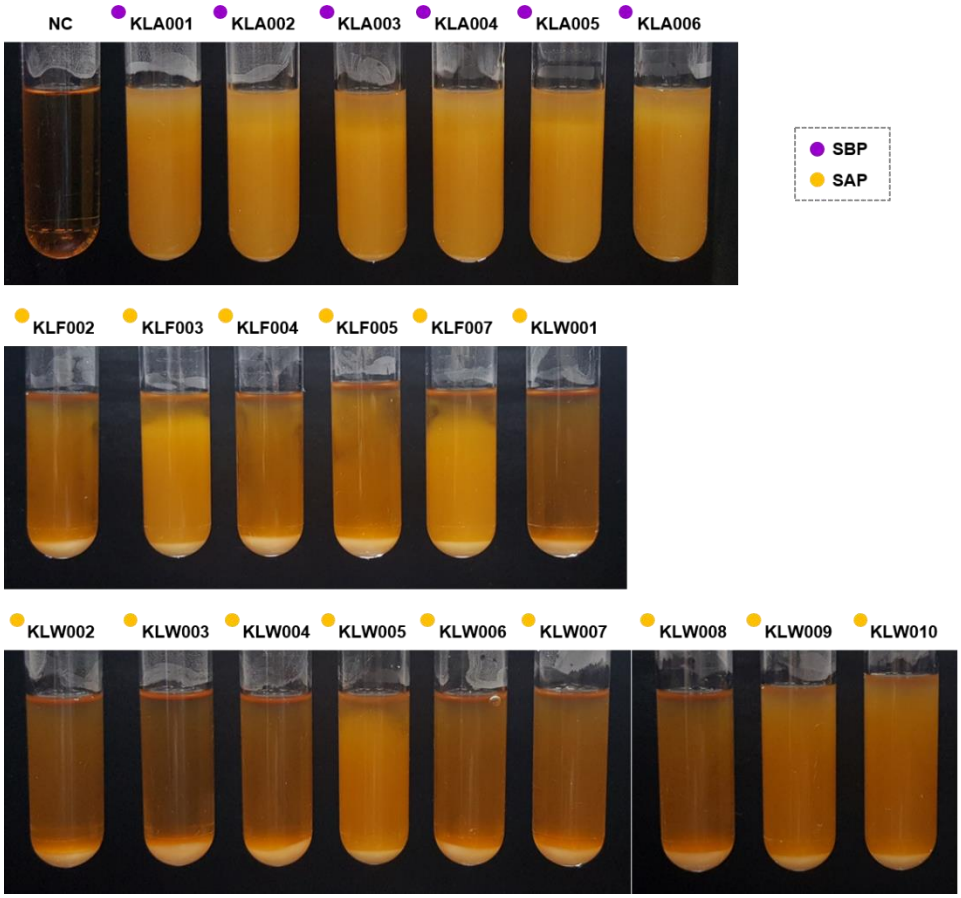


Figure 35. Difference in cell aggregation of SBPs and SAPs.

For this test, bacterial were cultured for 24 h at 37°C in shaking condition (240 rpm). Cell aggregation of the cultured bacteria was observed after incubation for 2 h at 37°C in static condition. NC, negative control (MRS broth only).

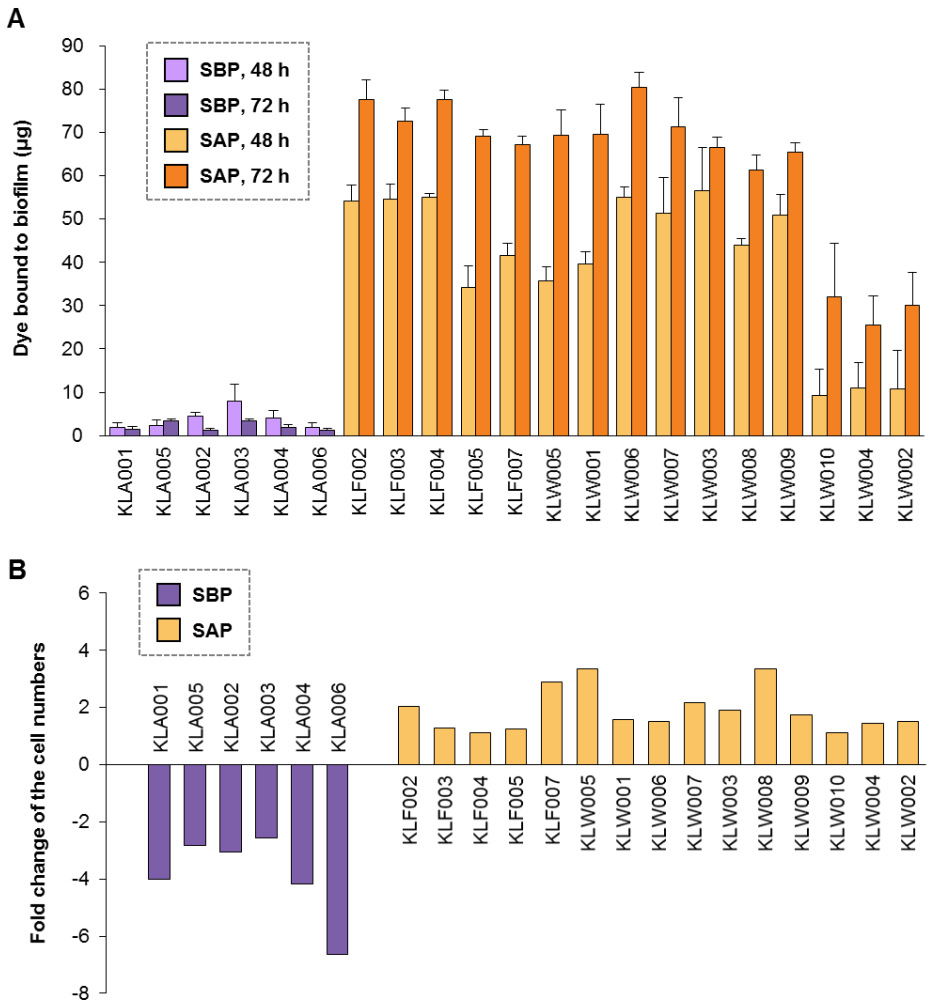


Figure 36. *In vitro* confirmation of the survival characteristics of *L. salivarius*.

(A) Detection of biofilm formation at 48 h and 72 h after incubation. (B) Fold change of the cell numbers of the isolated strains in competition with swine intestinal microbiota. The difference in the cell numbers between 24 h and 48 h after incubation was calculated.

4. Conclusion

To provide insight into the ecology and evolution of the probiotic species *L. salivarius* with their hosts, the draft genomes of 21 *L. salivarius* strains isolated from pigs were generated, and investigated the pan-genome of *L. salivarius*, focusing on their host specialization. A phylogenetic analysis based on MLSA highlights the host-specific lineages of *L. salivarius* that can be differentiated from other hosts, indicating that members of this species have been specialized to specific hosts for a long time. Commensal bacteria have increased their fitness in response to the selection pressure of their environments. The identification and comparison of several extracellular components, including some with mucus-binding domains and others with cell wall-anchoring domains, provides evidence that they are associated with adhesion and colonization. Host-specialized cell surface proteins play a crucial role in host adaptation, such as in the interaction with the epithelia of the animal GIT. Thus, probiotic bacteria are useless if the host specialization is not considered even though probiotics can provide benefits to their host. The distribution of the host-specific genes and their variants for cell surface components of *L. salivarius*, such as EPS and other proteins with extracellular domains, are distinguishable between hosts.

Other host-specific genes that are not associated with cell surface molecules were also found. The *L. salivarius* strains from the different hosts have evolved separately under distinct selective pressures, e.g., different host genotypes or

environmental factors such as dietary components. Interestingly, several host-specific genes not only contribute to bacterial growth but also provide beneficial effects to the hosts. This type of win-win interaction between the host and the microbe is termed as ‘mutualism’, which is important property of probiotic bacteria. Because these mutualistic traits are beneficial to both the host and microbe, hosts allow the colonization of these bacteria to occur and they in turn gain the advantages associated with surviving in the host habitat. In this interaction, the host and the commensal bacterium co-evolve, as has been described in many studies (Hooper and Gordon, 2001; Bäckhed et al., 2005). The host-specific traits related with these mutualistic properties will offer the valuable points for selection of probiotic microbes.

The *L. salivarius* strains isolated from humans have no host-specific genes for adhesion to the habitat and for energy production. As described above, the genome size of the human isolates is smaller than that of pig isolates. It is well documented that the evolution of commensal bacteria leads to the loss of many genes because a host provides various resources to commensals that free-living bacteria do not have access to (Toft and Andersson, 2010). This suggests that the human environment is more attractive than that of pigs and chickens for commensal bacteria. It is assumed that this is because the exposure frequency of humans to harsh environments, such as a disease, toxins and dangerous conditions is lower. The difference in the number of AR genes or choloylglycine hydrolase genes is further evidence of the distinct host environments.

In comparison between SBPs and SAPs, the investigation revealed the bacterial genomic dynamics affected by the AGP prohibition. After the prohibition, the intestinal environment colonized by gut microbiota was altered, and the intestinal bacteria evolved for adaptation to the changed habitat. Although many researchers showed that genomes of microorganisms are influenced by antibiotic selection pressure (Albrich et al., 2004; Davies and Davies, 2010; Fridman et al., 2014), there is limited evidence on the effect of antibiotic removal on the bacterial genome.

This genetic difference between SBPs and SAPs can be understood with two perspectives, individual evolution and population genetics. In aspect of individual evolution, the bacterial strains have acquired and lost several genes with DNA mutation for adaptation to the altered environment after the prohibition of AGPs. These changes in the genomes enable to form dense biofilm rapidly in the individual SAPs, and these strains had survived. Another perspective is population genetics, which deals with genetic differences within and/or between populations. It might have been that *L. salivarius* strains which have the ability for dense biofilm formation exist already before the ban of AGPs, although they are small population in the intestinal microbiota. In this situation, the bacteria that show less formation of biofilm such as SBPs would comprise the great majority of the *L. salivarius* population. As previously discussed it, because utilization of L-rhamnose, D-tagatose, and galactitol for energy production and nucleotide synthesis in SBPs could be helpful to protect them against antibiotics, the strains

that do not possess these sugar utilization ability occupy only small part of the *L. salivarius* population and eke out a living in the intestine. After the ban of AGPs, these strains might take the chances to be a major portion in the population due to their ability for dense and rapid biofilm formation, which contribute to increase the fitness in competition of the intestinal microbiota. The change of the bacterial portion in the *L. salivarius* population can be seen that 'repressed' ability for biofilm formation have been 'released' after the AGP prohibition.

Interestingly, a number of AR genes in the *L. salivarius* genomes were retained after AGP prohibition, although other genomic characteristics, such as genome size and biofilm formation-associated genes, were changed for adaptation to the environment that antibiotics are not ever-present. It is assumed that SAPs have no reason to discard AR genes after AGP prohibition. Antibiotics have been still used in animals for a veterinary purpose, and this situation made the bacteria keep AR genes in their genomes.

The host-specific traits of the bacteria identified in this study may provide a basis for future studies of *L. salivarius* and other mutualistic bacteria. Considering the functions of the host-specific genes, it seems that *L. salivarius* has evolved to adapt to hosts using three strategies; 1) increasing adhesion (and/or colonization) abilities to the environments such as epithelia, 2) producing more energy through changes in carbon utilization, 3) resistance against host stress such as bile detoxification with choloylglycine hydrolase. These are the most important traits for bacterial niche adaptation, and these factors must be considered when selecting

probiotics. It leaves much to be desired that the number of genomes of chicken *L. salivarius* strains is lack for extensive study. There are only three genomes of the chicken isolates. More isolation and sequencing of chicken *L. salivarius* strains are helpful to deeply investigate pan-genome of the species.

This report is the first to investigate the *L. salivarius* pan-genome and the effect of AGP prohibition on the changes in genomic features of animal intestinal bacterium based on genome sequencing. Although more studies are required to understand the relationship between biofilm formation and the genomic profile of *L. salivarius*, this study provides evidence that the ban of AGPs, which had been commonly added in feeds for growth promotion of livestock, can modify the bacterial genome to adapt to the altered microenvironment. The study provides a deeper understanding of the probiotic bacterium *L. salivarius*, and helps to characterize the ecology and evolution of *L. salivarius* with their hosts. Furthermore, given the many functional properties of *L. salivarius* as probiotic, the genetic features allowing dense biofilm formation can provide detailed insight for screening and selection of probiotic *L. salivarius* strains.

Study 2. Comparative Genomics of *Lactobacillus reuteri* Focusing on Host Adaptation and Antimicrobial Activity

1. Introduction

After the legal prohibition of AGPs, farm animals are vulnerable to attack by pathogens and the productivity ultimately decreased. The antipathogenic effect of probiotics as AGP alternatives is an important trait for the prevention of pathogenic infections. In this reason, *L. reuteri* and its antimicrobial compound reuterin are getting spotlight in the animal industry. *L. reuteri* is a heterofermentative lactic acid bacterium and gut symbiont colonizing epithelia in the GIT of mammals and birds (Casas and Dobrogosz, 2000; Walter, 2008) *L. reuteri* is an well-studied species to reveal its mode of action and interaction with hosts as a probiotic bacteria; such mechanisms include colonization of the intestine (Wadstroum et al., 1987; Valeur et al., 2004), prevention and treatment of diarrhea (Rosenfeldt et al., 2002; Francavilla et al., 2012), protection from pathogens (Reid and Burton, 2002; Spinler et al., 2008) and host immunomodulation (Niv et al., 2005; Borchers et al., 2009).

It was reported that reuterin produced by many *L. reuteri* strains shows broad-spectrum antimicrobial activity, and the probiotic properties of *L. reuteri* were

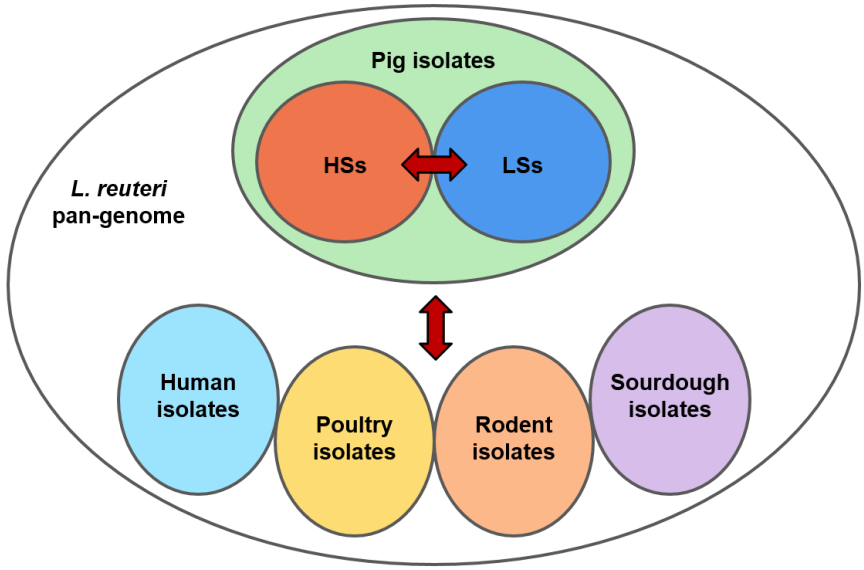
proposed to be related to the production of this antimicrobial compound (Lüthi-Peng et al., 2002; Vollenweider and Lacroix, 2004). Indeed, reuterin production is a major reason for the use of *L. reuteri* as a probiotic in animal feeds and in dairy products for human consumption (Vollenweider and Lacroix, 2004; Flint and Garner, 2009). Reuterin consists of 3-HPA, its hydrate, and its oligomers in dynamic equilibrium (Talarico and Dobrogosz, 1989; Vollenweider et al., 2003). 3-HPA is produced during the anaerobic metabolism of glycerol via the enzyme diol dehydrase (Talarico et al., 1988; Chung et al., 1989). However, some *L. reuteri* strains showed reduced antimicrobial activity and did not produce reuterin, and this difference in reuterin production has not been studied at the genomic level until now.

In this study, *L. reuteri* strains were isolated from porcine feces, and the antipathogenic effect of isolated *L. reuteri* strains was tested against two swine-related pathogens, *E. coli* K88 and *S.* Typhimurium. These pathogens are the most common causative microorganisms of diarrhea and sepsis in weaned pigs, and consequently cause critical damage in the pork industry (Baker et al., 1997; Quirke et al., 2001; Kim et al., 2010; Hur et al., 2011). 16 *L. reuteri* strains that showed either a higher or lower ability to produce reuterin were selected, and draft genomes of these strains were generated for comparative genomic analysis (Figure 37).

Although reuterin-producing *L. reuteri* is a good probiotic candidate for pigs, host specialization of *L. reuteri* also should be considered in determining its utility

as a porcine feed additive. Many studies have shown that *L. reuteri* strains are adapted to various host habitats (Lin and Savage, 1984; Oh et al., 2010; Frese et al., 2011). Their ability to adhere to host epithelial cells in the gut is also host-specific, because physiological characteristics of epithelia are dependent on a host species (Wesney and Tannock, 1979; Lin and Savage, 1984). For a deeper understanding of host adaptation of pig-origin strains, the pan-genome of *L. reuteri* was explored with 42 genomes, including the genomes generated in this study, focusing on swine-specific genetic features (Figure 37). This comparative genomic analysis will provide valuable insight into the mechanisms of host specialization and antimicrobial action of *L. reuteri*. Furthermore, this study will help to screen antipathogenic probiotics as an alternative to AGPs.

A



B

Study	Category	Item
-	Selection of the isolates	Measurement of antipathogenic activity
Pan-genome of *L. reuteri* - Host adaptation	General features	General genomic features
		Host origins
	Adhesion and colonization	Pig isolates-specific cell wall proteins
	Other features	Absent genes in the pig isolates
Antipathogenic activity - Genomic comparison of HSs and LSs	General features	Clustering HSs and LSs
	Functional difference	Difference in gene category
	Reuterin and cobalamin production	Whole genome comparison
		Gene cluster comparison
		Determination of HSs-specific genes
		Phylogeny of the gene cluster
	Citrate metabolism	Citrate metabolism in LSs
	In vitro validation	Identification of HSs-specific genes
		Development of a genetic marker

Figure 37. Graphical abstract of study 2.

(A) Organization of study 2. (B) Items observed in study 2.

2. Materials and Methods

1) Measurement of the antipathogenic effect of reuterin

The reuterin activity was measured against two pathogens (Figure 38). The isolated *L. reuteri* strains were inoculated into MRS broth containing 0.5% glycerol and cultivated at 37 °C for 24 hr. The cells were washed twice with 500 mM sodium phosphate buffer (pH 7.5). 3.8×10^7 cfu of each *L. reuteri* strain were incubated in 800 μl of 200 mM glycerol at 37 °C for 2 hr for reuterin production. Each supernatant was filtered with a syringe filter (0.45 μm diameter) and plated in triplicate on a 96-well plate. Next, 1.5×10^6 cfu of *E. coli* K88 or *S.* Typhimurium were added to the wells. The plates were incubated at 37 °C for 24 hr, and the absorbance at 600 nm for each well was measured in a microplate reader (Infinite M200 Pro, Tecan, Zürich, Switzerland). The antipathogenic effect of each strain against each pathogen was validated with three replicate measurements.

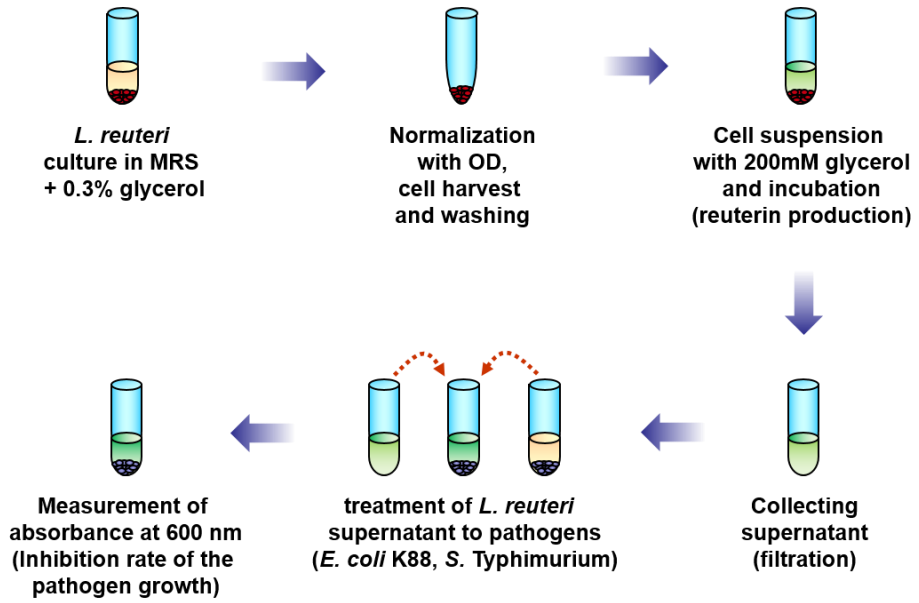


Figure 38. A method for measurement of reuterin activity.

L. reuteri cells were incubated with glycerol, which is a source of reuterin. After then, the supernatant was collected and treated to the pathogens to detect inhibition of the pathogenic growth.

2) Generation of draft genomes of *L. reuteri* strains

L. reuteri strains were cultured in MRS broth at 37 ℃ for 24 hr, and bacterial cells were harvested. *L. reuteri* gDNA was extracted using the G-spin Total DNA Extraction Kit (Intron Biotechnology, Seongnam, Republic of Korea) according to a standard protocol. A gDNA library for Illumina sequencing was constructed with ~350 bp inserts using Nextera XT DNA Library Preparation Kit (Illumina, CA, USA) according to the manufacturer's recommendations. The prepared *L. reuteri* gDNA libraries were then sequenced using HiSeq 2500 (Illumina, CA, USA) for 100 bp paired-end reads.

After the sequencing, adapter sequences of the reads were trimmed with Cutadapt 1.10 (Martin, 2011), and the sequence reads were quality-filtered using in-house Perl scripts (Kim and Marco, 2014). In brief, when 95% of the nucleotide bases in a read were given a quality score over 31 (Illumina 1.8+) and the read length was ≥ 70 bp, the read was used for *de novo* genome assembly. The filtered paired reads were assembled using SPAdes 3.9 (Bankevich et al., 2012). ANIb was calculated by JSpeciesWS with default options (Richter et al., 2016). To test clonality between the generated genomes, the nucleotide sequences of the polymorphic sites in the core genomes were collected and compared. The degree of completion of the generated genomes was tested by aligning the sequencing reads to the draft genome. Alignment of the short paired reads was carried out using Bowtie2 with the default options and portion of the mapped reads per total reads was represented (Langmead and Salzberg, 2012).

3) Genome collection and ortholog identification

All *L. reuteri* genomes available at the NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/genomes/438) were collected for exploration of the *L. reuteri* pan-genome (in September 2016, Table 8). The 16 genomes generated in this study and 26 NCBI genomes were annotated by RAST with default options. The presence of RAST subsystems in a genome was used for hierarchical clustering using the Euclidean distance method implemented in R software (Team, 2013). Existence of subsystems was statistically examined by 1,000 bootstrapping using an R package, Pvclust (Suzuki and Shimodaira, 2006).

For identification of orthologous CDSs, all CDSs of the 16 draft genomes and 26 NCBI genomes were collected, and orthologs were identified as previously described (Figure 10) (Kim and Marco, 2014). Briefly, CDSs in the annotations were filtered to remove CDSs containing premature stop codons (pseudogenes). Each CDS was then aligned to the entire CDS pool using GASSST 1.28, according to nucleotide sequence identity ($\geq$ 85%) and best sensitivity (Rizk and Lavenier, 2010). The aligned CDSs were regarded as one ortholog, and the consensus sequence of each ortholog was determined using the CAP3 with default options (Huang and Madan, 1999).

Table 8. *L. reuteri* genomes obtained from NCBI database for this study.

Strain	Host	MLSA group	Geographical origin	NCBI accession
ATCC53608	Swine	IV	Sweden	CACS00000000
I5007	Swine	IV	China	NC_021494
ZLR003	Swine	IV	China	NZ_CP014786
CRL1098	Sourdough	II	Germany	LYWI00000000
LTH2584	Sourdough	III	Germany	JOSX00000000
LTH5448	Sourdough	I	Germany	JOOG00000000
TMW1.112	Sourdough	III	Germany	JOKX00000000
TMW1.656	Sourdough	III	Germany	JOSW00000000
100-23	Rodent	III	New Zealand	AAPZ00000000
480_44	Rodent	I	Israel	MBLQ00000000
482_46	Rodent	I	Israel	MBLR00000000
482_54	Rodent	I	Israel	MBLS00000000
484_32	Rodent	I	Israel	MBLT00000000
484_39	Rodent	I	Israel	MBLU00000000
I49	Rodent	III	Switzerland	NZ_CP015408
lpuph	Rodent	I	USA	AEAX00000000
mlc3	Rodent	III	USA	AEAW00000000
TD1	Rodent	I	USA	NC_021872
P43	Poultry	I	USA	MCNS00000000
CF48-3A	Human	VI	Finland	ACHG00000000
DSM20016	Human	II	Germany	NC_009513
IRT	Human	II	South Korea	NZ_CP011024
JCM1112	Human	II	Germany	NC_010609
MM2-3	Human	II	N.D.	ACLB00000000
MM4-1A	Human	II	N.D.	ACGX00000000
SD2112	Human	VI	N.D.	NC_015697

*N.D., Not determined.

4) Hierarchical clustering and functional analysis of genes

All phylogenetic analyses based on nucleotide sequences were carried out using MEGA7 (Kumar et al., 2016). The nucleotide sequences were retrieved through global alignment of orthologous CDSs from each genome, and were compared using the multiple sequence alignment software MUSCLE 3.8.31 (Edgar, 2004). The phylogenetic relationship was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are reported in number of base substitutions per site. Clustering based on MLSA used the nucleotide sequences of the seven *L. reuteri* genes, *ddl, pkt, leuS, gyrB, dltA, ropA,* and *recA* (Oh et al., 2010). Genomic alignment for circular comparison of the genomes was carried out using BRIG with default options (Alikhan et al., 2011).

5) Identification of genes *in vitro*

PCR primers for amplification of the *L. reuteri* genes were designed using Primer3Plus (Untergasser et al., 2012). Primers were selected considering conserved sequences of the genes, especially four nucleotides at 3' end of the primers. All primers used in this study are shown in Table 9. 1 μl of forward and reverse primers (10 pmole, respectively) were mixed with 10 ng of gDNA and 10 μl of i-Taq 2x PCR master mix solution (Intron Biotechnology, Seongnam, Korea), and distilled water was added to reach a reaction volume of 20 μl. PCR was performed as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 sec, 55 °C for 1 min, 72 °C for 1 min; 72 °C for 5 min.

For multiplex PCR, eight primers were used including six primers used in the gene identification; hemB_2R, cbiP_6R, cbiO_2F, cbiO_3R, sirA_4F and sirA_3R. Two primers were newly designed for detection of multi-band in gel electrophoresis; hemB_6F and cbiP_6F. Information of primer sets and size of the amplicons used in the multiplex PCR were provided in Table 10.

Table 9. PCR primer sets for identification of the *L. reuteri* genes.

Gene	Primer name	Nucleotide sequence (5'-3')	Start position	Length (bp)	Tm (ºC)	GC content (%)	Product size (bp)
cbiD	cbiD_4F	TGGYGGYGATGAYCARGATG	246	20	59.9	55	445
	cbiD_1R	YTTRGCRAARTCYTCWCCAT	690	20	60	55	
cbiO	cbiO_2F	AGGACTWCGWAAWYTRCGGC	219	20	60.1	55	461
	cbiO_3R	ARACGKTTTSCYTATYTCCC	679	20	57.3	50	
cbiP	cbiP_3F	AYTCGGAARYCRCGWCAAAA	721	20	60.2	50	416
	cbiP_6R	GCAACSGCCTGWGTTGTTGT	1136	20	60.4	55	
hemB	hemB_2F	TACYGGTCACTGYGGAATTC	375	20	60.1	55	558
	hemB_2R	ATTAAGTCTGCYCCAGCTCG	932	20	59.8	55	
hemD	hemD_2F	TGGCGRATRMRRTTAGAACA	46	20	59.8	50	565
	hemD_3R	AMTTWCCSGTRYTYKYTCCT	610	20	59.8	50	
sirA	sirA_4F	GGATWCMRGATAWKTATCRGCGG	71	23	59.1	47.8	226
	sirA_3R	GCMAYACTKGGWGAYTTTCCC	296	21	60	52.4	
Ortholog_120	120_F	AAGGYGGTAATCAAAGGGCT	64	20	59.7	50	518
	120_R	TGTCCTTCACGCAAGAGCTT	581	20	59.9	50	
Ortholog_1236	1236_F	CGAAAAACCAGTTGGGCCAA	36	20	59.5	50	309
	1236_R	GCCAATAAGCCTGCAACTCC	344	20	59.5	55	
Ortholog_3044	3044_F	TGGACCCCAGCACAAAAGTT	733	20	60	50	550
	3044_R	CTGGACGACCTTGCGTTACT	1282	20	60	55	

Ortholog_3565	3565_F	TATGATGGCGTTGTGGTTGC	4	20	59.2	50	513
	3565_R	ACTGGTCGTATCCGGGGTAA	516	20	60	55	
Ortholog_4217	4217_F	TGGTCGGTCTAGCGAGCATA	2	20	60.5	55	101
	4217_R	GCCAGCTTCGTCCATTACCT	102	20	60.1	55	
Ortholog_5421	5421_F	TGCCATTTATCATGTCACGGG	11	21	58.7	47.6	325
	5421_R	ACACCCCAACTACTAATGTCATCA	335	24	59.5	41.7	
Ortholog_331	331_F	AACGGTGCTCAGGTACTTGG	82	20	60	55	582
	331_R	TGGATGCTGTTGGAGAGCTG	663	20	60	55	
Ortholog_827	827_F	TGTTGCTCAAGAAAGTGGCC	54	20	59	50	309
	827_R	TCAGCACTCTCTTTTAATCGCCT	362	23	60.1	43.5	
Ortholog_3325	3325_F	TTTGGTAACCTCGGGCACTC	58	20	60	55	542
	3325_R	TGCCGATGATGACCTGTAGC	599	20	59.9	55	
Ortholog_4560	4560_F	AATCTGTGCGCGTGTTTGTC	207	20	60	50	529
	4560_R	ACTTTCTTTTGCCCGGGCTA	735	20	59.9	50	
Ortholog_4830	4830_F	TGGATAAATACACCGTTGAATTAAGTG	2	27	58.3	33.3	101
	4830_R	TTTAGCGTCGTTCATTGCCT	102	20	58.2	45	
Ortholog_5258	5258_F	TGGAACCCTCCGCAATAAGT	42	20	59	50	506
	5258_R	TCTGTAAAGCTATGGTCTTGGCA	547	23	59.7	43.5	

Table 10. Primer sets for the multiplex PCR.

Gene	Primer name	Nucleotide sequence (5'-3')	Start position	Length (bp)	Tm (°C)	GC content (%)	Product size (bp)
hemB	hemB_6F*	GTTGCAATTTGATCGTCATCGTCG	3	24	58.6	45.83	930
	hemB_2R	ATTAAGTCTGCYCCAGCTCG	932	20	59.8	55	
cbiP	cbiP_6F*	TTAATATGGGGATGGCSCGAATG	446	23	58.9	47.83	691
	cbiP_6R	GCAACSGCCTGWGTTGTTGT	1136	20	60.4	55	
cbiO	cbiO_2F	AGGACTWCGWAAWYTRCGGC	219	20	60.1	55	461
	cbiO_3R	ARACGKTTTSCYTATYTCCC	679	20	57.3	50	
sirA	sirA_4F	GGATWCMRGATAWKTATCRGCGG	71	23	59.1	47.8	226
	sirA_3R	GCMAYACTKGGWGAYTTTCCC	296	21	60	52.4	

* These primers were newly designed for the multiplex PCR.

3. Results and Discussion

1) Antipathogenic activity of the isolated *L. reuteri* strains

The *L. reuteri* strains that inhabited the GIT of pigs were isolated from several farms in the Republic of Korea. 634 LAB colonies from porcine feces were isolated, and among them, 104 *L. reuteri* colonies were identified by *L. reuteri*-specific PCR. After isolation and identification, the antipathogenic effect of reuterin produced by isolated *L. reuteri* strains was measured against *E. coli* K88 and *S.* Typhimurium, which cause diarrhea and sepsis in swine (Baker et al., 1997; Quirke et al., 2001; Kim et al., 2010; Hur et al., 2011). It was found that *L. reuteri* strains displayed a broad range of bacteriocidal action (Figure 39). 16 strains were selected for comparative genomic analysis, including the 8 strains showing the higher antipathogenic effect and the 8 strains showing the lower effect.

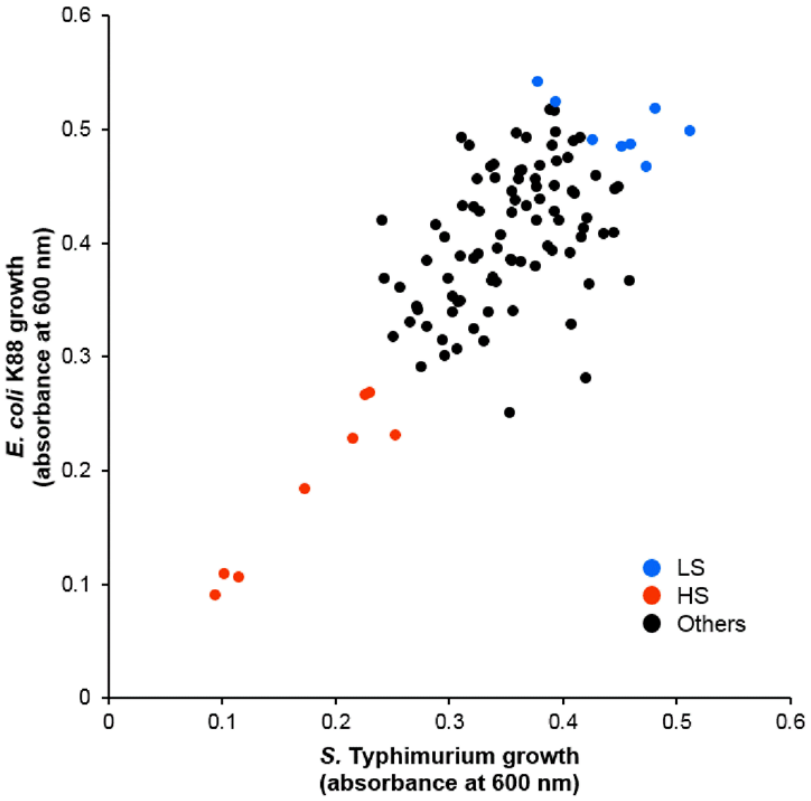


Figure 39. Antimicrobial activity of isolated *L. reuteri* strains against *E. coli* K88 and *S*. Typhimurium.

The horizontal and vertical axes mean the growth of each pathogen. Red dots, strains showing higher antimicrobial activity (group HS); blue dots, strains showing lower antimicrobial activity (group LS); black dots, other *L. reuteri* strains isolated in this study.

2) Pan-genome of *L. reuteri*

(1) Genomic features of the isolated *L. reuteri* strains

Draft genomes of the selected 16 *L. reuteri* strains were obtained by Illumina high-throughput sequencing and *de novo* assembly (Table 11). Each draft genome was compared to the reference genome, *L. reuteri* DSM20016, by measuring the ANIb. The strains showed over 95% ANIb, confirming their identities as members of the same species, *L. reuteri* (Zheng et al., 2015). The slightly lower ANIb values of just over 95% for the isolated strains reflected their host specialization (humans and pigs). The degree of completion of draft genomes was measured by aligning the sequencing reads to the draft genome. All genomes reflect over 97% of mapped reads as shown in Table 11. Clonality of the generated genomes was tested by confirmation of polymorphic sites in the core genomes of the 16 *L. reuteri* strains. Total 40,971 polymorphic sites were found and the hierarchical clustering based on the polymorphic sites showed the distinction of the 16 strains (Figure 40). These results indicated that the *L. reuteri* strains are genetically different strains from each other.

Isolated strains had an average genome size of 2.11 ± 0.19 Mbp, 1,954.56 ± 169.98 CDSs and 38.67 ± 0.09% GC content. There were no significant differences between group HS and LS (genome size, 2.13 ± 0.26 Mbp vs. 2.10 ± 0.11 Mbp; number of CDSs, 1966.38 ± 222.61 vs. 1942.75 ± 109.71; GC content, 38.63 ± 0.09% vs. 38.70 ± 0.0% in group HS and LS, respectively).

Table 11. Genomic feature of *L. reuteri* strains generated in this study.

Group	Strain	Sampling farm	Genome size (Mbp)	No. of contigs	Max. contig size (Kbp)	N50	No. of CDSs	GC%	ANIb* (%)	Read depth (fold)	Mapped reads (%)	NCBI accession
LS	KLR1001	A	2.06	145	119.53	25,287	1,894	38.59	96.17	413.34	99.05	MIME00000000
LS	KLR1002	A	2.66	392	79.53	19,985	2,383	38.50	95.63	237.93	98.94	MIMF00000000
LS	KLR2001	B	2.11	149	151.45	38,417	2,011	38.53	95.94	294.55	99.41	MIMI00000000
LS	KLR2004	C	1.95	141	155.31	33,810	1,804	38.65	95.73	204.45	99.13	MIML00000000
LS	KLR2006	D	1.91	96	134.08	38,836	1,756	38.64	96.16	313.99	98.46	MIMN00000000
LS	KLR3002	E	1.99	228	98.17	36,298	1,827	38.76	95.88	236.57	99.46	MIMR00000000
LS	KLR3003	E	1.97	142	151.93	35,675	1,843	38.75	95.95	261.77	99.16	MIMS00000000
LS	KLR3006	F	2.37	257	79.06	20,771	2,213	38.63	95.27	223.69	99.39	MIMV00000000
HS	KLR1004	A	2.20	154	85.18	27,988	2,065	38.52	95.42	287.95	98.81	MIMH00000000
HS	KLR2002	B	2.13	169	75.70	23,306	1,971	38.73	95.62	279.45	98.67	MIMJ00000000
HS	KLR2003	B	2.08	149	110.82	23,303	1,950	38.70	95.49	237.11	97.66	MIMK00000000
HS	KLR2007	D	1.98	136	69.78	26,881	1,814	38.71	95.59	280.69	99.67	MIMO00000000
HS	KLR2008	D	1.98	143	69.78	24,137	1,809	38.71	95.58	252.54	99.69	MIMP00000000
HS	KLR3004	F	2.00	149	73.75	23,580	1,837	38.75	95.66	328.03	99.00	MIMT00000000
HS	KLR3005	F	2.28	172	109.91	32,119	2,066	38.82	95.38	303.36	98.94	MIMU00000000
HS	KLR4001	C	2.14	136	104.85	30,962	2,030	38.67	95.29	256.00	97.54	MIMW00000000

* Average nucleotide identity based on BLAST+ to the reference strain, *L. reuteri* DSM20016.

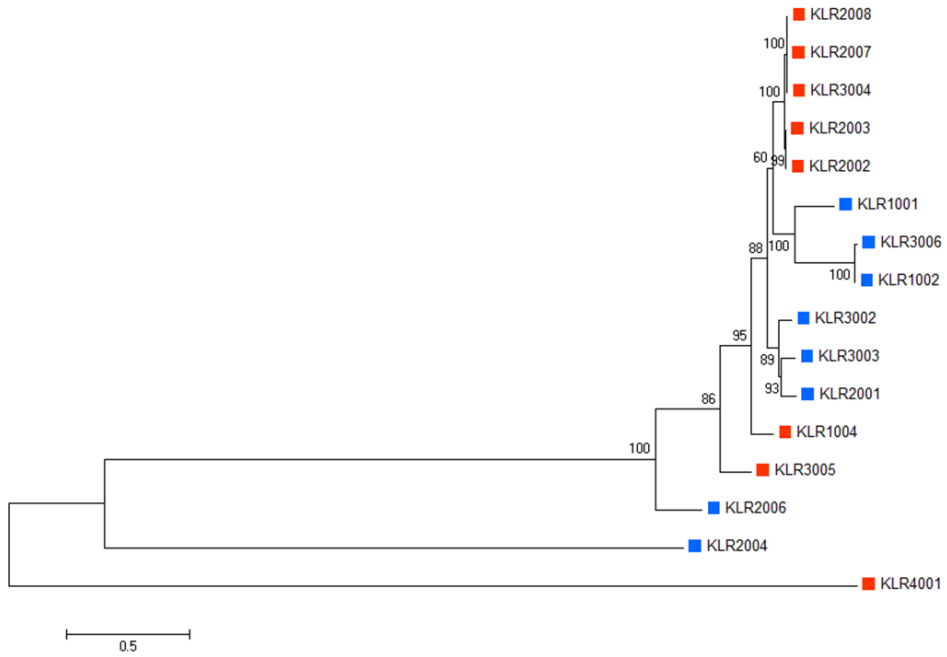


Figure 40. Hierarchical clustering of 16 *L. reuteri* genomes based on the polymorphic sites.

The genomes generated in this study were clustered based on the nucleotide sequence of the polymorphic sites in the core genomes. Red box, HS; blue box, LS.

(2) Classification of *L. reuteri* genomes by their hosts

Commensal microbes in the animal gut play roles in the development and performance of hosts (Dethlefsen et al., 2007), and they coevolved with their hosts through reciprocal interaction (Ley et al., 2008b). *L. reuteri* is also evolved for niche adaptation and provides a beneficial effect to the host. *L. reuteri* strains were divided based on their hosts in phylogenetic clusters with MLSA and AFLP (Oh et al., 2010), and some host-specific traits were identified (Lin and Savage, 1984; Oh et al., 2010; Frese et al., 2011). To obtain a deeper understanding of host adaptation of pig-origin strains, the *L. reuteri* pan-genome from 42 *L. reuteri* genomes was explored. 19 *L. reuteri* clade IV strains, including ATCC53608, ZLR003 and I5007, were compared with strains originating from different hosts: 7 human strains, 10 rodent strains, 5 sourdough strains and one poultry strain (Table 8 and Table 11).

The 42 collected genomes were phylogenetically clustered using MLSA (Figure 41). It is well-documented that *L. reuteri* clusters based on seven housekeeping genes can be divided into six clusters by their isolation host, and pig-origin *L. reuteri* strains belong to cluster IV and V (Oh et al., 2010; Su et al., 2012). There was no strain from cluster V among the 26 genomes in the NCBI genome database and group HS and LS, and the 16 strains isolated in this study were grouped into *L. reuteri* cluster IV. The geographical origin of the strains was not associated with the MLSA clades as reported previously (Oh et al., 2010), and group HS and LS were not divided into two lineages in this phylogenetic analysis.

Genomic annotation by RAST and collection of pan-genome orthologs revealed that there are 6,520 orthologs in *L. reuteri* pan-genome, containing 2,187 hypothetical protein genes. The *L. reuteri* pan-genome possesses 890 core genes and 2,081 unique genes (Figure 42A), and clade IV (pigs) strains possess 1,338 core genes and 1,110 unique genes (Figure 42B). Interestingly, the human isolates possessed 1,373 core genes and only 88 unique genes. Although JCM1112 and DSM20016 are derived from the same isolate (*L. reuteri* F275), the two genomes share 432 genes, as shown in (Figure 42D). The number of human core genes is similar between pigs and rodent (1,347 genes, Figure 42C), but fewer unique genes are present in human strains compared with swine, rodent (1055 genes) and sourdough (983 genes, Figure 42E). The genomic variety of porcine strains was similar to that of rodents, and human strains possessed a less diverse gene composition, in agreement with a previous study (Frese et al., 2011). Swine, rodent and human strains have a comparable number of orthologs per strain, but the genomes of the human strains possess fewer unique genes, and certain genes, such as *ureC* and *pduC*, are present in all or none of the human strains. It seems that the human gut may require more specific genetic features and a stricter standard for genome composition of their commensal bacteria than other hosts.

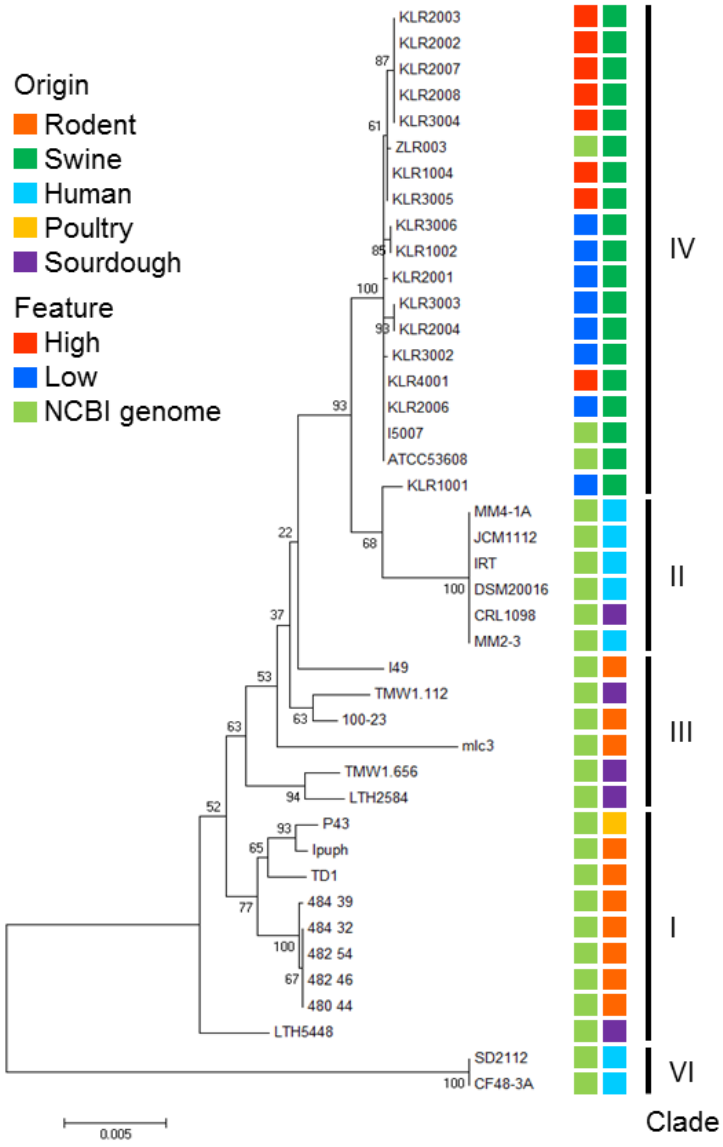


Figure 41. Hierarchical clustering of 42 *L. reuteri* genomes based on MLSA.

The clade of L. reuteri varies in composition from I to VI, but there is no strain belonging to clade V among the 42 strains used in this study. The bootstrap consensus tree inferred from 1,000 replicates is taken.

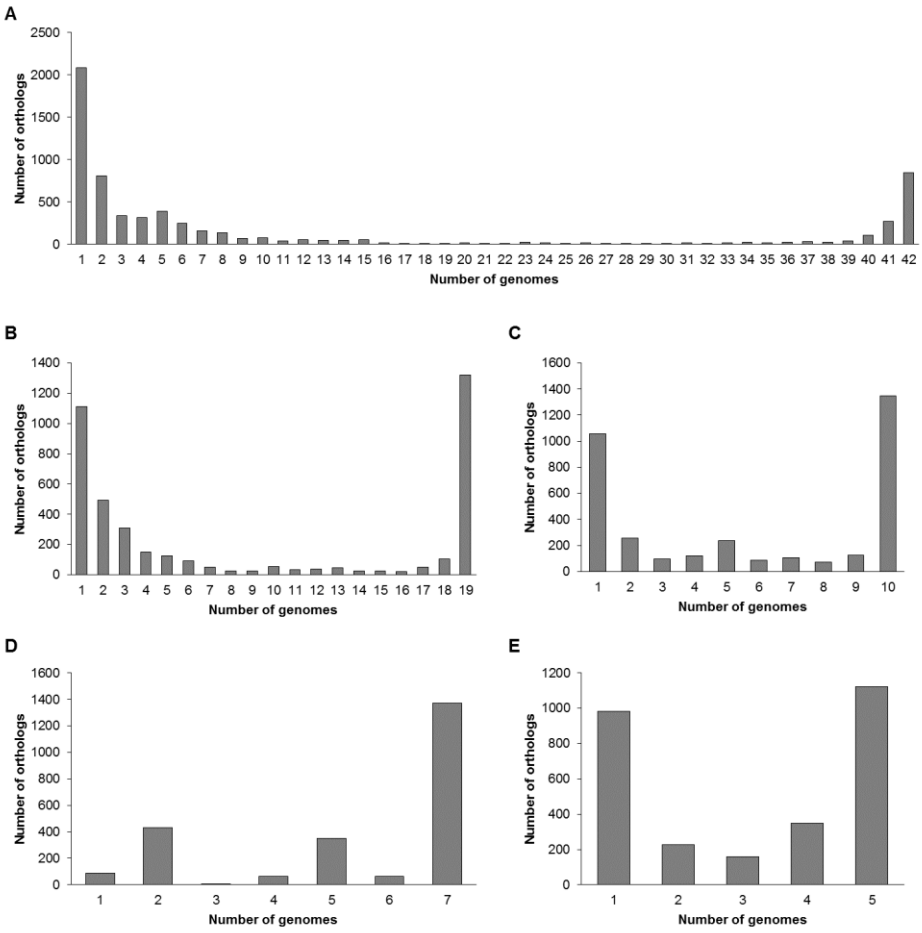


Figure 42. Distribution of orthologous CDSs in *L. reuteri* pan-genome.

Distribution histograms are shown for 42 *L. reuteri* genomes (A). (B) 19 swine strains. (C) 10 rodent strains. (D) 7 human strains. (E) 5 sourdough strains. The horizontal axis indicates the number of isolates sharing the same orthologous CDSs, and vertical axis represents the number of orthologous CDSs shared by the indicated number of isolates.

(3) Host-specific traits of the porcine *L. reuteri* strains

The result of phylogenetic clustering based on RAST subsystems is different from MLSA clustering (Figure 43). Porcine strains were gathered in one cluster, although the other strains isolated from rodent, human, poultry and sourdough were dispersed and not categorized for each host, indicating that porcine strains have host-specific features. Although there was no significant difference between the subsystems of porcine strains and other strains, six clade IV-specific genes consisting of three hypothetical protein genes and three annotated genes were identified *in silico* and *in vitro* (Table 12 and Figure 44). Among them, ortholog_1236 and ortholog_3044 encode cell surface proteins, which may have an important role in *L. reuteri* clade IV for colonization of the porcine gut epithelia. There are many reports demonstrating that cell surface proteins of intestinal lactobacilli contribute to host adaptation, because each host species has specialized gastrointestinal epithelia. For example, the stomachs of swine and rodent are lined by a nonglandular squamous stratified epithelium, which is adhered and colonized by lactobacilli, whereas the human stomach is lined by mucosa composed of glandular epithelia (Tannock, 1992).

Several evidences suggesting that the host-specific genes of the porcine strains were obtained by HGT were found. Transposase genes are neighbors of the ortholog_1236 and ortholog_3044 (Figure 45A and B). Another clade IV-specific gene ortholog_120 encodes transposase, and surface proteins, such as the LPXTG-motif cell wall anchor domain protein, are located upstream of

ortholog_120 in porcine strains (Figure 45C). This co-localization of transposases and surface protein genes indicates that the surface protein genes might be acquired by HGT. Furthermore, other genes located at flanking regions of clade IV-specific genes are also highly conserved. The *dlt* operon is responsible for D-alanylation of several compounds such as TA, which is a component of the cell surface polymer of Gram-positive bacteria, and this operon is located upstream of ortholog_120. The *dap* operon, which synthesizes L-lysine from L-aspartate, is located upstream of ortholog_1236, and ortholog_3044 is next to ABC transporter genes. These proximal gene sets were also identified in other species, especially in pathogenic bacteria such as *Escherichia*, *Salmonella*, *Listeria* and *Streptococcus*. *htpX* and *lemA* homologs located downstream of ortholog_120 were also identified in several pathogens. This inter-specific homology of the genes implied the possibility of acquisition of the clade IV-specific genes and their neighboring genes from other bacteria. The different GC content of these genetic loci as compared to the average genomic GC content also provides evidence of HGT (Lawrence and Ochman, 2002). The GC content of poorly conserved regions differs from the average GC content of bacterial genomes, because the GC content differed between donor and recipient bacteria. Nevertheless, some loci are highly conserved in clade IV despite having a fluctuated GC content that differs from the average of the genome (Figure 46), implying that these genes would increase bacterial fitness and perform critical roles for survival in the porcine gut. An intensive study is needed to determine whether the clade IV strains has acquired the genes from other bacteria or not. Another possibility cannot be excluded that

these genes are originally existed in the genomes of the clade IV and these strains are the donors of the genes to other bacteria including pathogens.

It was found that six genes were absent in the pig isolates but present in certain other strains (Table 12 and Figure 44). Two genes encode hypothetical proteins, and four genes are annotated by RAST. The annotated genes were associated with several pathways of energy production and/or nucleotide metabolism, which are important processes for bacterial life, but the results showed that the genes are not essential for the growth and survival of *L. reuteri* porcine strains. This may reflect the differences in living conditions, such as diet, among host species, and genomes of commensal bacteria may have evolved accordingly, although further study is needed.

Table 12. Clade IV-specific genes and absent genes in clade IV compared with other *L. reuteri* clades.

Ortholog_ID	Product (nomenclated by RAST)	Locus tag (NCBI genomes)
Clade IV-specific genes		**in ATCC53608**
Ortholog_120	Mobile element protein	LRATCC53608_1775
Ortholog_1236	Gram-positive anchor domain	LRATCC53608_1334
Ortholog_3044	Cell wall surface anchor family protein	LRATCC53608_0212
Ortholog_3565	Conserved hypothetical protein	LRATCC53608_0617
Ortholog_4217	Hypothetical protein	LRATCC53608_1955
Ortholog_5421	Hypothetical protein	LRATCC53608_1738
Absent genes in clade IV		**in DSM20016**
Ortholog_331	Glucosamine-6-phosphate deaminase (EC 3.5.99.6)	Lreu_1026
Ortholog_827	Conserved hypothetical protein	Lreu_1028
Ortholog_3325	Lactose and galactose permease, GPH translocator family	Lreu_1086
Ortholog_4560	Pyridine nucleotide-disulphide oxidoreductase family protein	Lreu_1310
Ortholog_4830	Conserved hypothetical protein	Lreu_1598
Ortholog_5258	Filamentation induced by cAMP protein Fic	Lreu_1027

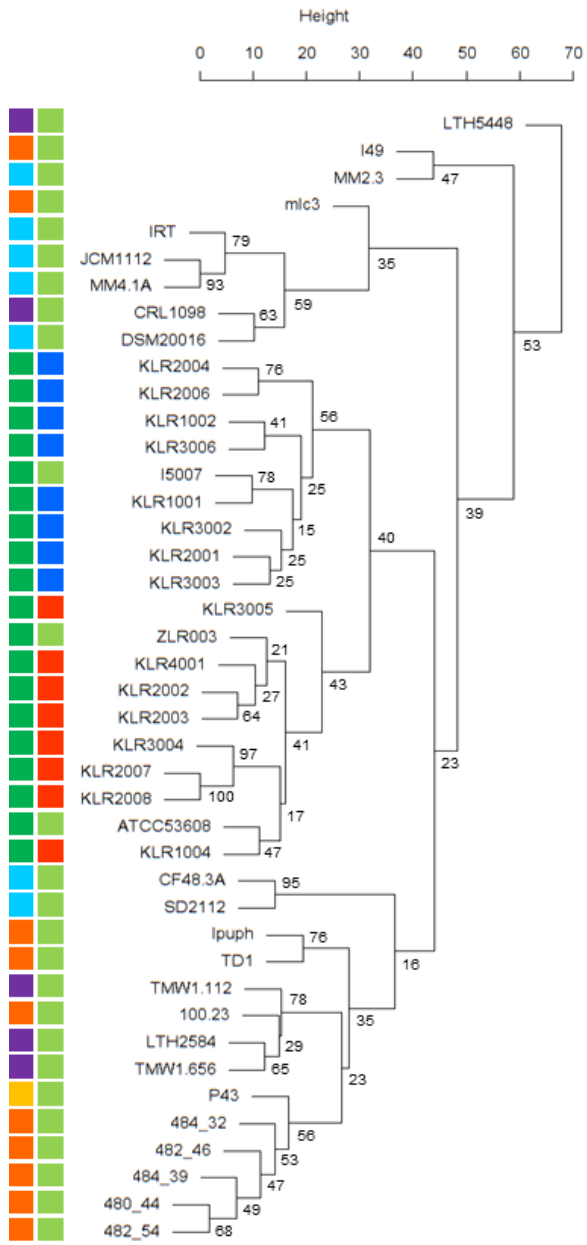


Figure 43. Hierarchical clustering of 42 *L. reuteri* genomes based on functional features.

The distribution of RAST subsystems was used for this analysis. The bootstrap consensus tree inferred from 1,000 replicates is taken.

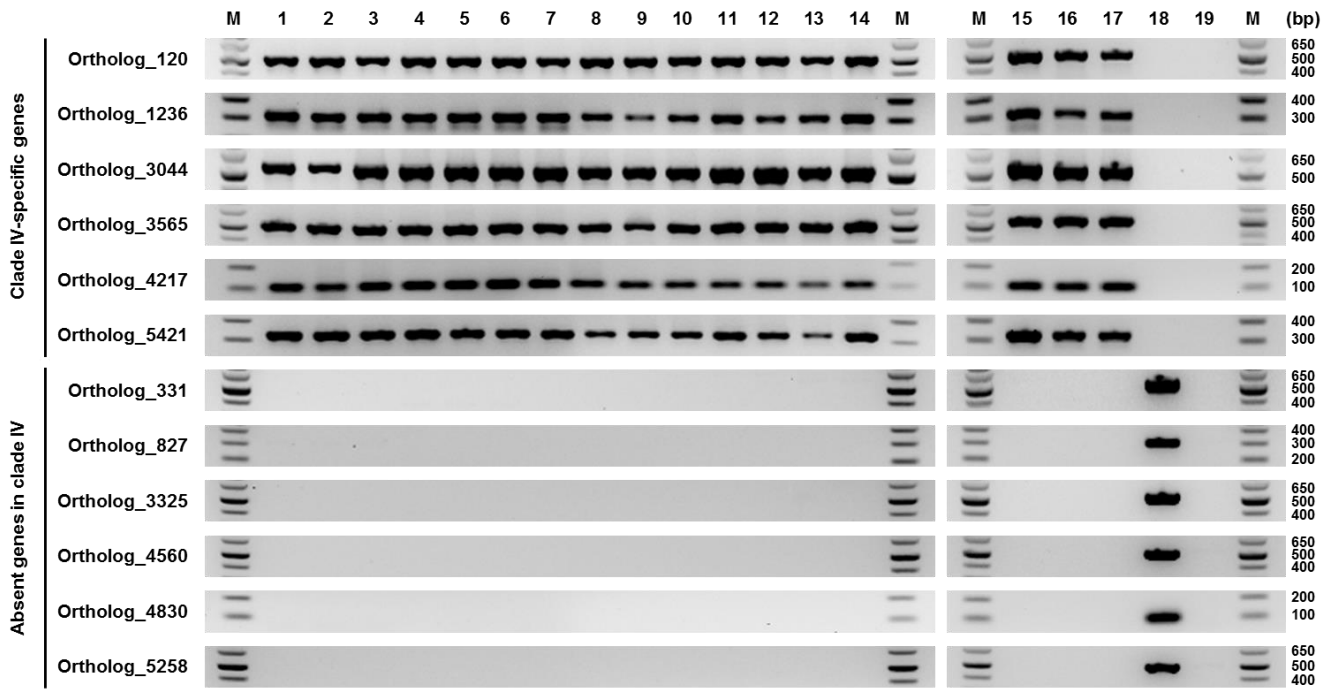
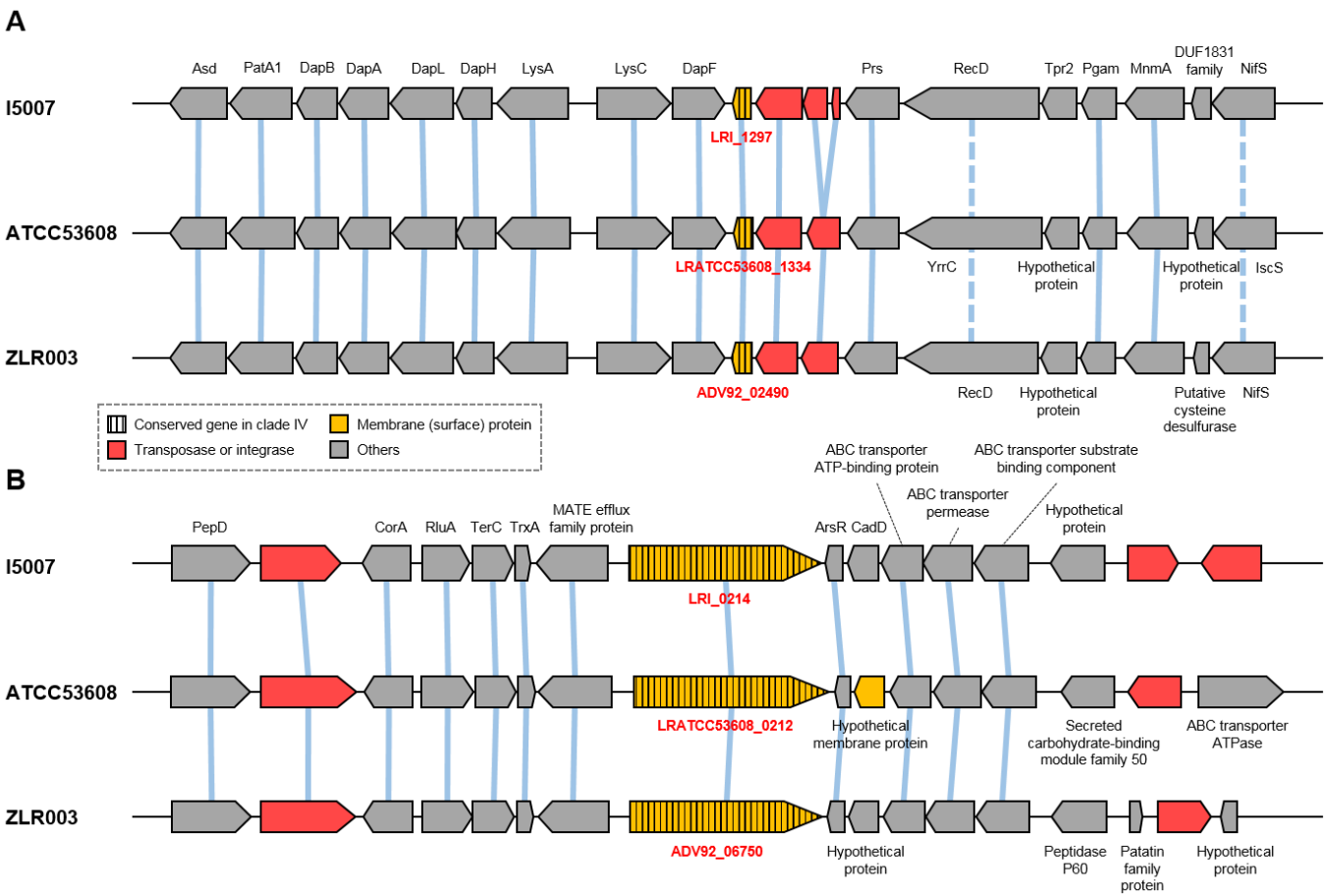


Figure 44. PCR identification of the clade IV-specific genes and absent genes in clade IV.

M, DNA molecular weight marker; 1, ATCC53608; 2, KLR1004; 3, KLR2002; 4, KLR2003; 5, KLR2007; 6, KLR2008; 7, KLR3004; 8, KLR3005; 9, KLR4001; 10, KLR1001; 11, KLR1002; 12, KLR2001; 13, KLR2004; 14, KLR2006; 15, KLR3002; 16, KLR3003; 17, KLR3006; 18, DSM20016; 19, Negative control.


A
Asd
PatA1
DapB
DapA
DapL
DapH
LysA
LysC
DapF
Prs
RecD
Tpr2
Pgam
MnmA
DUF1831 family
NifS
I5007
LRI_1297
ATCC53608
LRATCC53608_1334
YrrC
Hypothetical protein
Hypothetical protein
IscS
ZLR003
ADV92_02490
RecD
Hypothetical protein
Putative cysteine desulfurase
NifS
Conserved gene in clade IV
Membrane (surface) protein
Transposase or integrase
Others
B
ABC transporter ATP-binding protein
ABC transporter substrate binding component
ABC transporter permease
Hypothetical protein
PepD
CorA
RluA
TerC
TrxA
MATE efflux family protein
ArsR
CadD
I5007
LRI_0214
ATCC53608
LRATCC53608_0212
Hypothetical membrane protein
Secreted carbohydrate-binding module family 50
ABC transporter ATPase
ZLR003
ADV92_06750
Hypothetical protein
Peptidase P60
Patatin family protein
Hypothetical protein

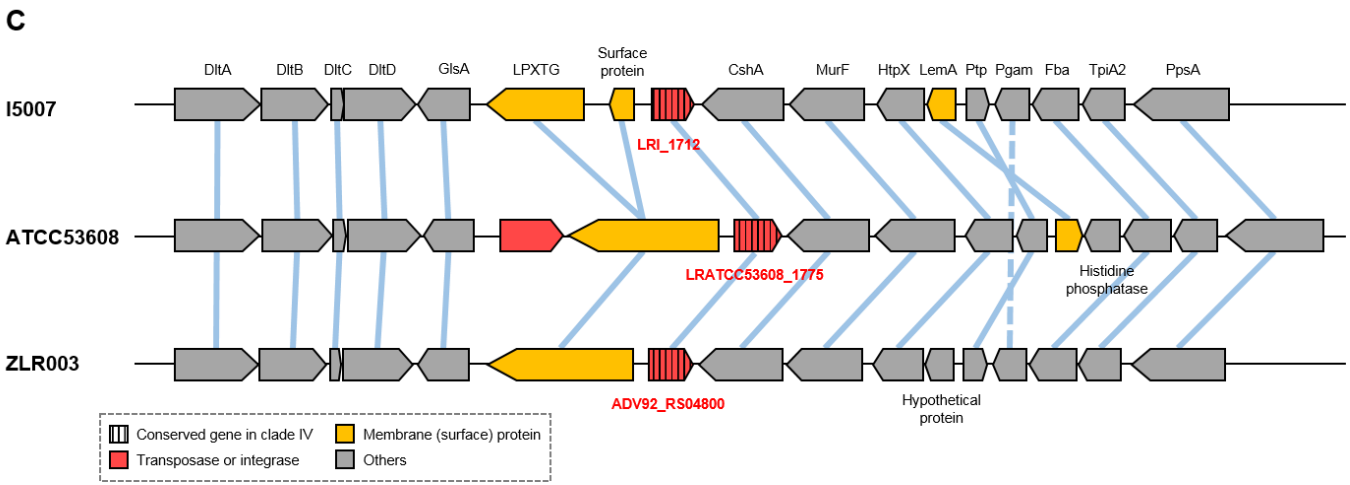


Figure 45. Neighboring genes located within 10 kb of both the flanking regions of *L. reuteri* clade IV-specific orthologs in *L. reuteri*.

Ortholog_1236 (A), ortholog_3044 (B) and ortholog_120 (C) in three clade IV strains were presented. Azure line indicates genes with same annotated function. Abbreviations; Asd, aspartate-semialdehyde dehydrogenase; PatA1, amino acid aminotransferase A1; DapB, dihydrodipicolinate reductase; DapA, dihydrodipicolinate synthase; DapL, LL-diaminopimelate aminotransferase; DapH, 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase; LysA, diaminopimelate decarboxylase; LysC, lysine-sensitive aspartokinase 3; DapF, diaminopimelate epimerase; Prs, ribose-phosphate pyrophosphokinase; RecD, exodeoxyribonuclease V subunit alpha; YrrC, ATP-dependent RecD-like DNA helicase; Tpr2, tetratricopeptide repeat protein 2;

Pgam, phosphoglycerate mutase; MnmA, tRNA-specific 2-thiouridylase; DUF, domain with unknown function; NifS, cysteine desulfurase; PepD, peptidase D; CorA, magnesium transporter; RluA, RNA pseudouridine synthase; TerC, tellurium resistance protein; TrxA, thioredoxin 1; ArsR, transcriptional regulator in ars operon; CadD, cadmium resistance permease; ABP, ATP-binding protein in ABC transporter; DltA, D-alanine--poly(phosphoribitol) ligase subunit 1; DltB, D-alanyl transfer protein; DltC, D-alanine--poly(phosphoribitol) ligase subunit 2; DltD, extramembrane protein DltD involved in dlt operon; GlsA, glutaminase A; LPXTG, cell wall-anchoring protein with LPXTG domain; CshA, DEAD-box ATP-dependent RNA helicase; MurF, UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate-D-alanyl-D-alanine ligase; HtpX, protease HtpX; LemA, membrane protein LemA; Ptp, protein tyrosine phosphatase; Fba, fructose-bisphosphate aldolase; TpiA2, triosephosphate isomerase 2; PpsA, phosphoenolpyruvate synthase.

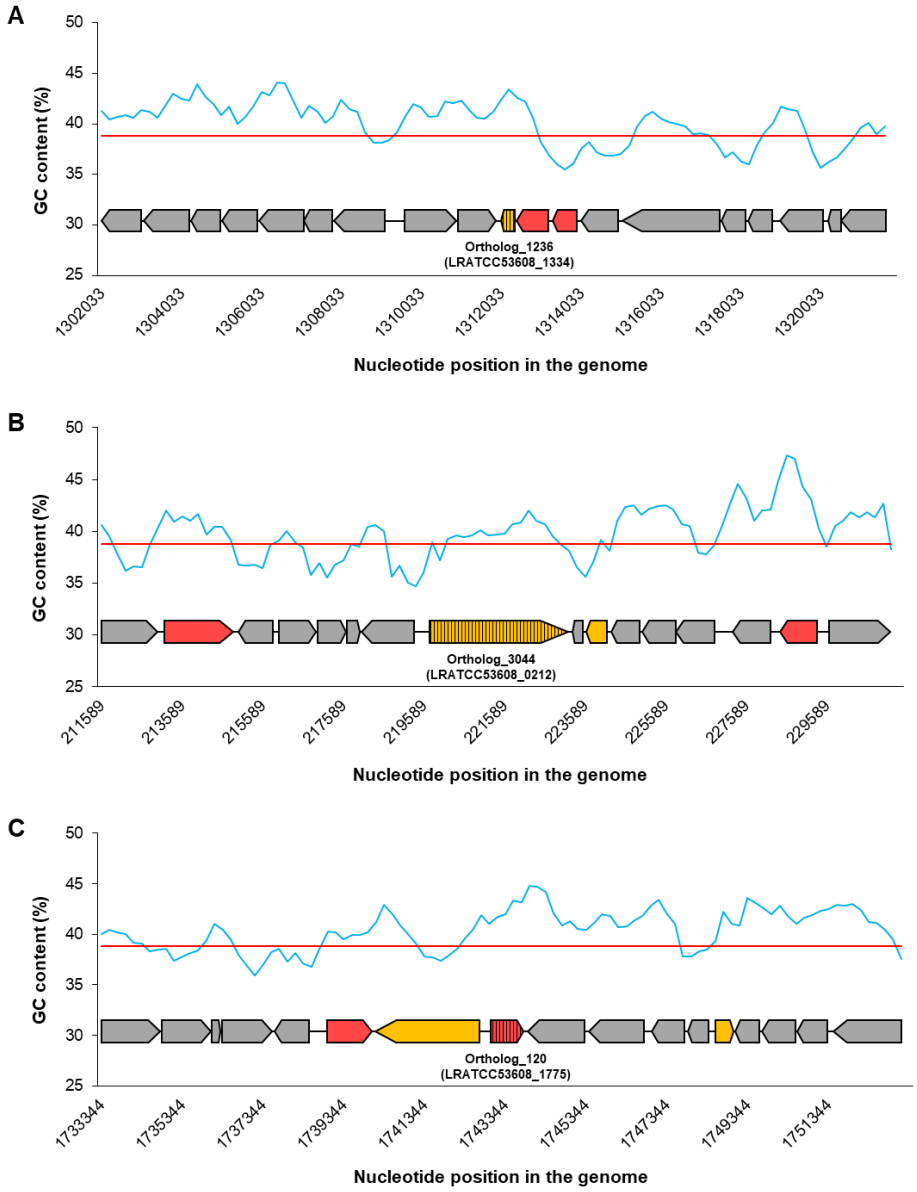


Figure 46. GC contents of neighboring genes of *L. reuteri* clade IV-specific orthologs in *L. reuteri* ATCC53608.

Ortholog_1236 (A), ortholog_3044 (B) and ortholog_120 (C) and their neighboring genes were presented as Figure 45. Red line means average GC content of the strain (38.78%). GC content was analyzed with window = 1,000 and step = 200.

3) *L. reuteri* strains isolated in this study

(1) Functional comparison of group HS and LS

As described above, hierarchical clustering based on RAST subsystems revealed that 19 porcine *L. reuteri* strains were distinguishable from the strains isolated from other hosts, and group HS formed a distinct lineage from LS in the clustering (Figure 43). In this phylogenetic tree, ATCC53608 and ZLR003 were included in group HS, and I5007 belonged to group LS. ATCC53608 had effective bacteriocidal ability in the presence of glycerol, and this was due to reuterin production (Muthukumarasamy et al., 2003), suggesting that ATCC53608 also has the same genetic property for antibacterial activity as group HS. The statistical analysis for RAST subsystems revealed that subsystem Cobalamin Synthesis was absent in group LS ($P = 1.15 \times 10^{-38}$), and subsystem Transport of Nickel and Cobalt was inexistent in 7 of the 8 strains of group LS ($P = 4.41 \times 10^{-7}$) (Figure 47). Group HS possessed more genes for subsystem Heme and Siroheme Biosynthesis than group LS, which has fewer or no genes of this subsystem ($P = 7.95 \times 10^{-7}$). Additionally, *L. reuteri* KLR1002, KLR2004, KLR2006 and KLR3006 in group LS contained subsystem Citrate Metabolism, Transport, and Regulation in their genomes, although group HS did not possess these genes ($P = 2.49 \times 10^{-9}$). Other subsystems showed significant differences between the groups, but these subsystems were found infrequently among the strains.

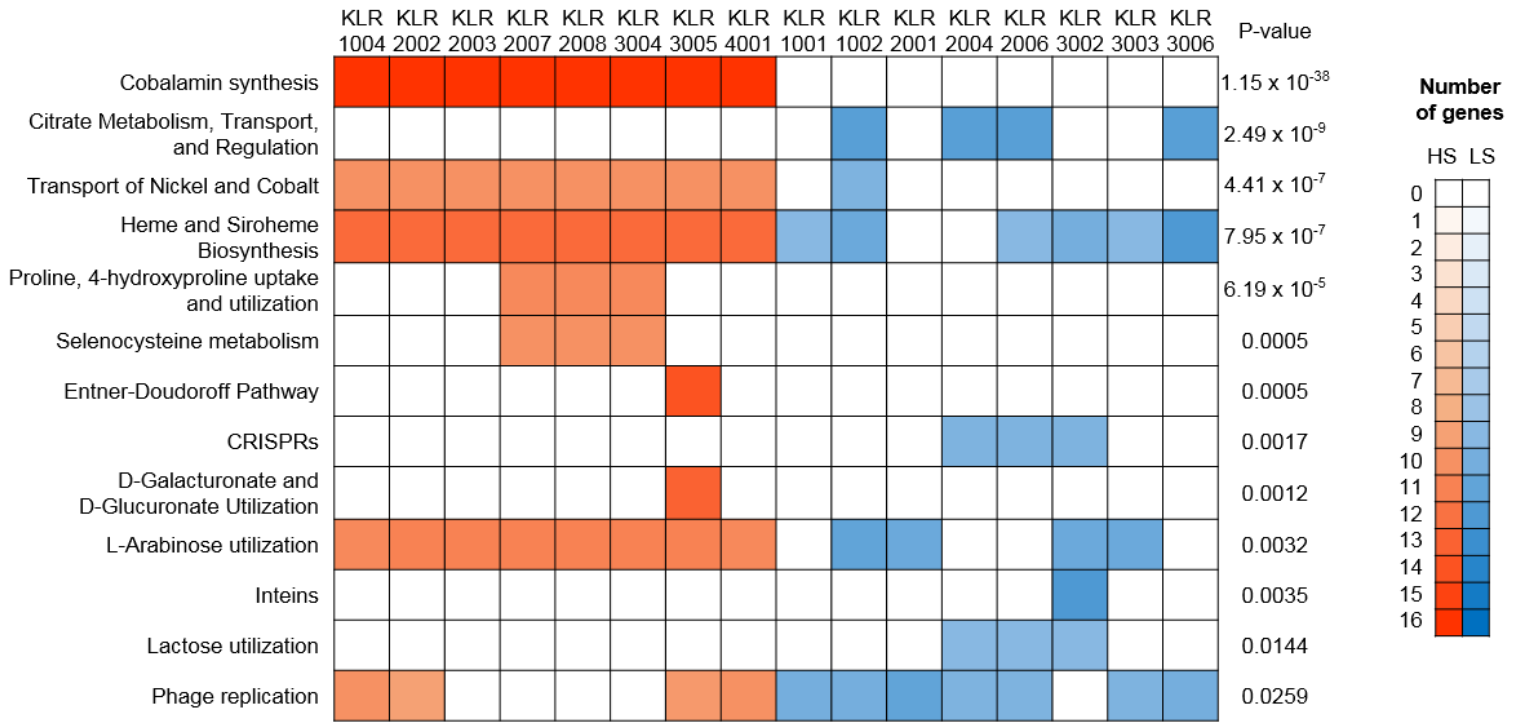


Figure 47. Comparison of functional features between group HS and LS.

The distribution of genes involved in RAST subsystems was shown. Statistical analysis was carried out using Fisher's exact test, and the significantly different subsystems in the two groups were showed ($P < 0.05$).

(2) Differences in reuterin and cobalamin production between the two groups

Three subsystems, Cobalamin Synthesis, Transport of Nickel and Cobalt, and Heme and Siroheme Biosynthesis, are responsible for cobalamin and reuterin synthesis by *L. reuteri*. Reuterin production is an important trait for *L. reuteri* as a probiotic for the inhibition of pathogen growth; however, some *L. reuteri* strains, such as those in group LS, do not have this property. Reuterin production is regulated by the *pdu-cbi-cob-hem* cluster of 58 genes in the *L. reuteri* genome (Morita et al., 2008). This cluster consists of 20 *pdu* genes, 17 *cbi* genes, 6 *cob* genes, 5 *hem* genes and 10 accessory genes. *pdu* genes are responsible for synthesizing 3-HPA from glycerol, and *cbi*, *cob* and *hem* genes are required for cobalamin biosynthesis. Cobalamin is essential for reuterin production, because diol dehydrase, which converts glycerol to 3-HPA, is a cobalamin-dependent enzyme (Talarico et al., 1988; Chung et al., 1989).

The genetic difference in the *pdu-cbi-cob-hem* cluster between group HS and LS was also observed when their genomes were aligned to the genomes of ATCC53608, ZLR003 and the reference strain DSM20016 (I5007 did not possess this gene cluster, Figure 48). The cluster was present in all strains of group HS, but absent or partially present in group LS. Among the three swine-origin NCBI genomes, ATCC53608 and ZLR003 also possessed this gene cluster, and this result matched the clustering based on RAST subsystems. This result demonstrated that presence of the *pdu*, *cbi*, *cob* and *hem* genes led to the distinct

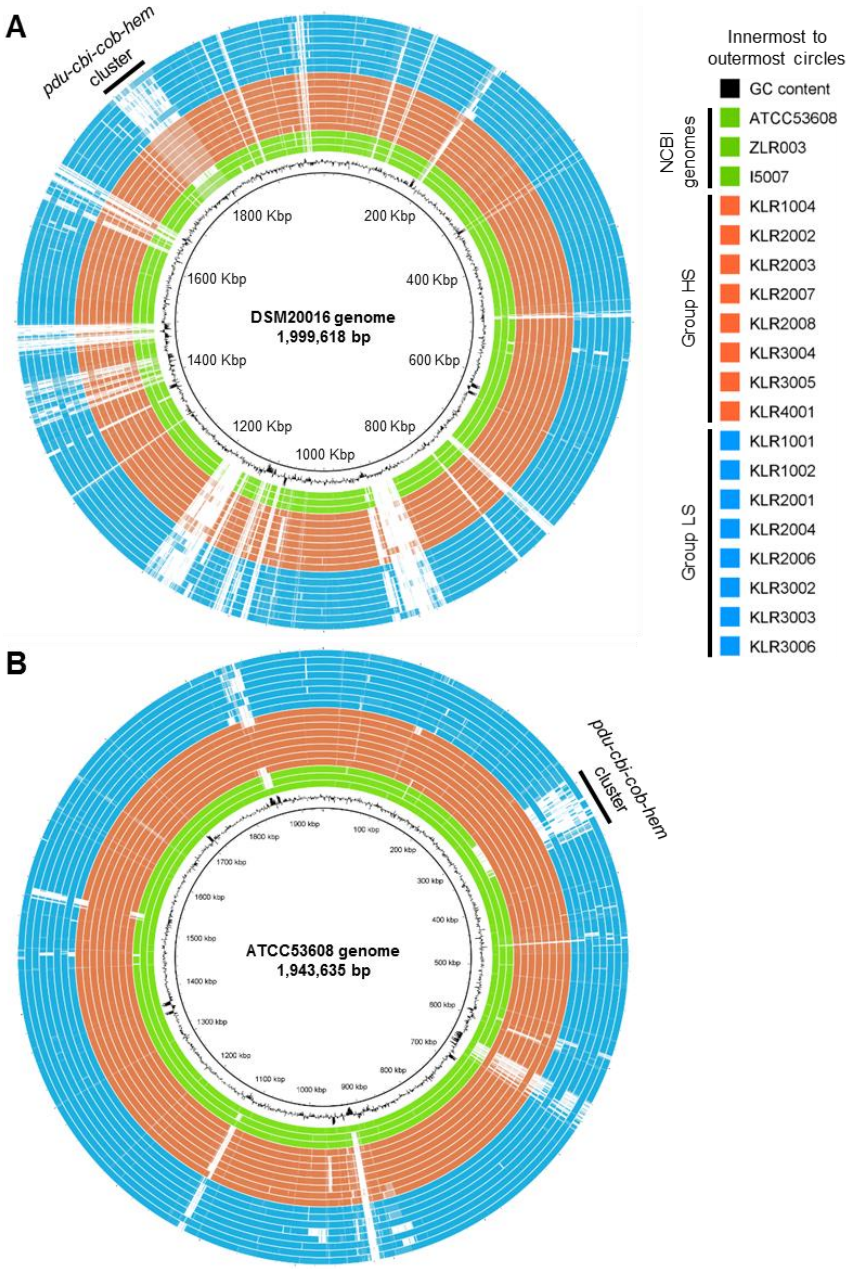
branching in subsystem-based hierarchical clustering between group HS and LS.

LS strains did not show bacteriocidal activity and *cbi*, *cob* and *hem* genes were partially or completely absent in 8 strains of group LS, although two LS strains possessed all *pdu* genes (Figure 49). This indicates that cobalamin synthesis genes are essential for higher antimicrobial activity, even if *pdu* genes are present in the *L. reuteri* genome. ATCC53608 and ZLR003 also had all genes of the cluster, similar to group HS, but I5007 had only two genes, *pduF* (locus tag LREU_RS09130 in the reference strain DSM20016) and *tra8* (LREU_RS08990), which were conserved in all porcine strains. *pduF* is a transporter that allows the cellular uptake of glycerol. Glycerol is not only used as a source of 3-HPA but is also converted to dihydroxyacetone phosphate via glycerol-3-phosphate or dihydroxyacetone, and dihydroxyacetone phosphate is utilized in the glycolytic pathway to produce energy. Therefore, even if reuterin and cobalamin are not produced in *L. reuteri*, glycerol and its uptake by *pduF* are important for growth and survival, and this explains why the *pduF* gene remains in all *L. reuteri* genomes.

The transposase gene *tra8* is present in all porcine *L. reuteri* strains and is located in the boundary between the *pdu* and *cbi-cob-hem* genes. This cluster had a lower GC content in comparison to the genomic average (36.51% versus 38.90% in DSM20016). Furthermore, ISs were identified within 20 kb of both the flanking regions of the cluster in the *L. reuteri* genome (Morita et al., 2008). This cluster is not common in other lactobacilli, but *pdu*, *cbi*, *cob* and *hem* genes were identified

in certain pathogenic genera, such as *Salmonella, Yersinia, Listeria, Streptococcus* and *Shigella* (Morita et al., 2008), similar to the porcine-specific genes. Interestingly, the hierarchical clustering based on MLSA showed that the difference in the *pdu-cbi-cob-hem* cluster between groups is not due to host adaptation, and the subsystem comparison also showed the same result. Any group-specific features contributing to the colonization or attachment to the porcine GIT were not found in group HS or LS, although this cluster was acquired by HGT.

Two strains of group LS, KLR1002 and KLR3006, have all of the *pdu* genes, similar to group HS, but the strains did not express the antipathogenic effect. As many studies have described previously, *pdu-cbi-cob* and *hem* genes were inserted together into the *L. reuteri* genome, and this process was mediated by transposons. Considering this fact, it is possible that some genes of the cluster may have been lost after the insertion by HGT, and it is obvious that *pdu-cbi-cob-hem* genes are not essential for survival in the porcine intestine. This unnecessariness is also observed in rodent *L. reuteri* strains, although all human strains had this gene cluster (Frese et al., 2011).


A
pdu-cbi-cob-hem cluster
1800 Kbp
200 Kbp
1600 Kbp
400 Kbp
DSM20016 genome
1,999,618 bp
1400 Kbp
600 Kbp
1200 Kbp
800 Kbp
1000 Kbp
Innermost to outermost circles
GC content
NCBI genomes
ATCC53608
ZLR003
I5007
Group HS
KLR1004
KLR2002
KLR2003
KLR2007
KLR2008
KLR3004
KLR3005
KLR4001
Group LS
KLR1001
KLR1002
KLR2001
KLR2004
KLR2006
KLR3002
KLR3003
KLR3006
B
pdu-cbi-cob-hem cluster
ATCC53608 genome
1,943,635 bp

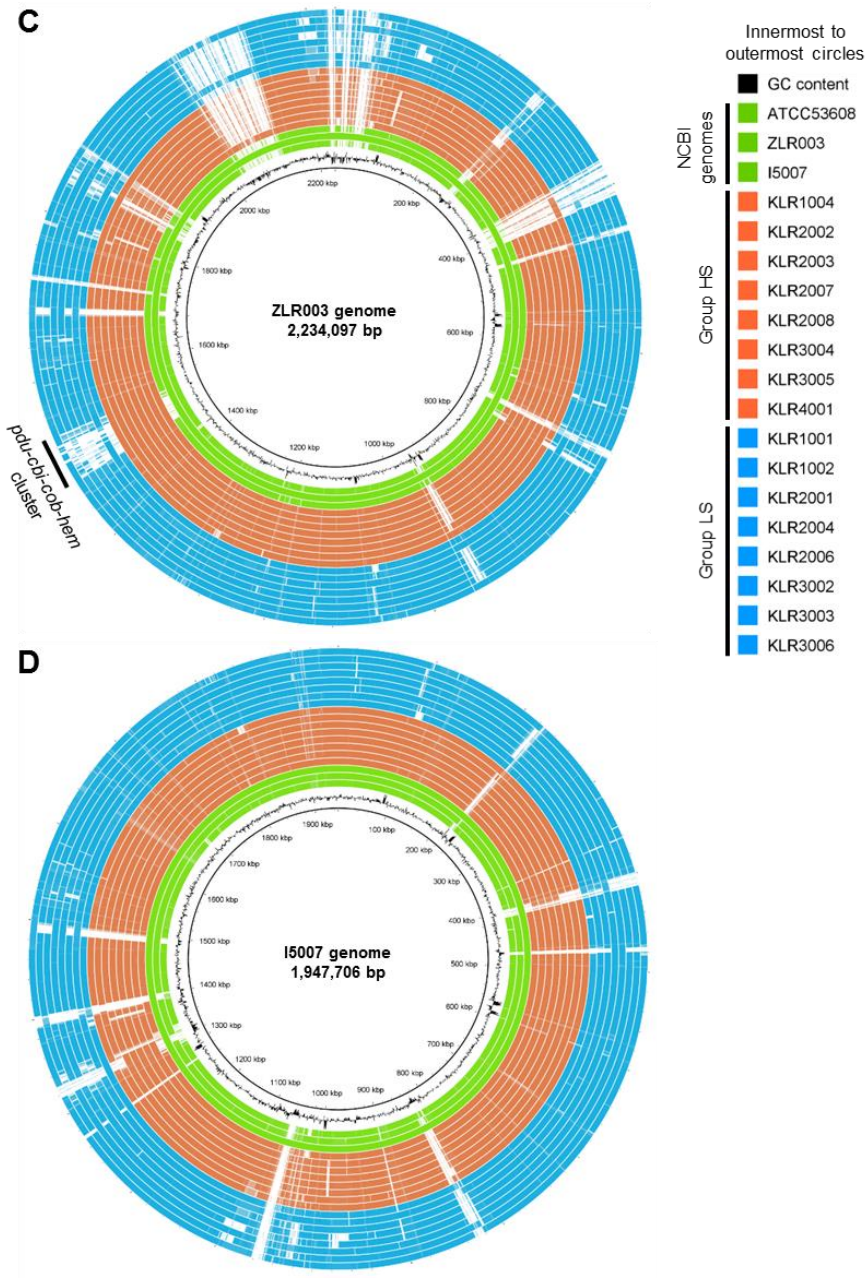


Figure 48. Circular comparison of 19 porcine *L. reuteri* genomes.

The genomes were aligned to the reference genome DSM20016 (A), ATCC53608 (B), ZLR003 (C) and I5007 (D).

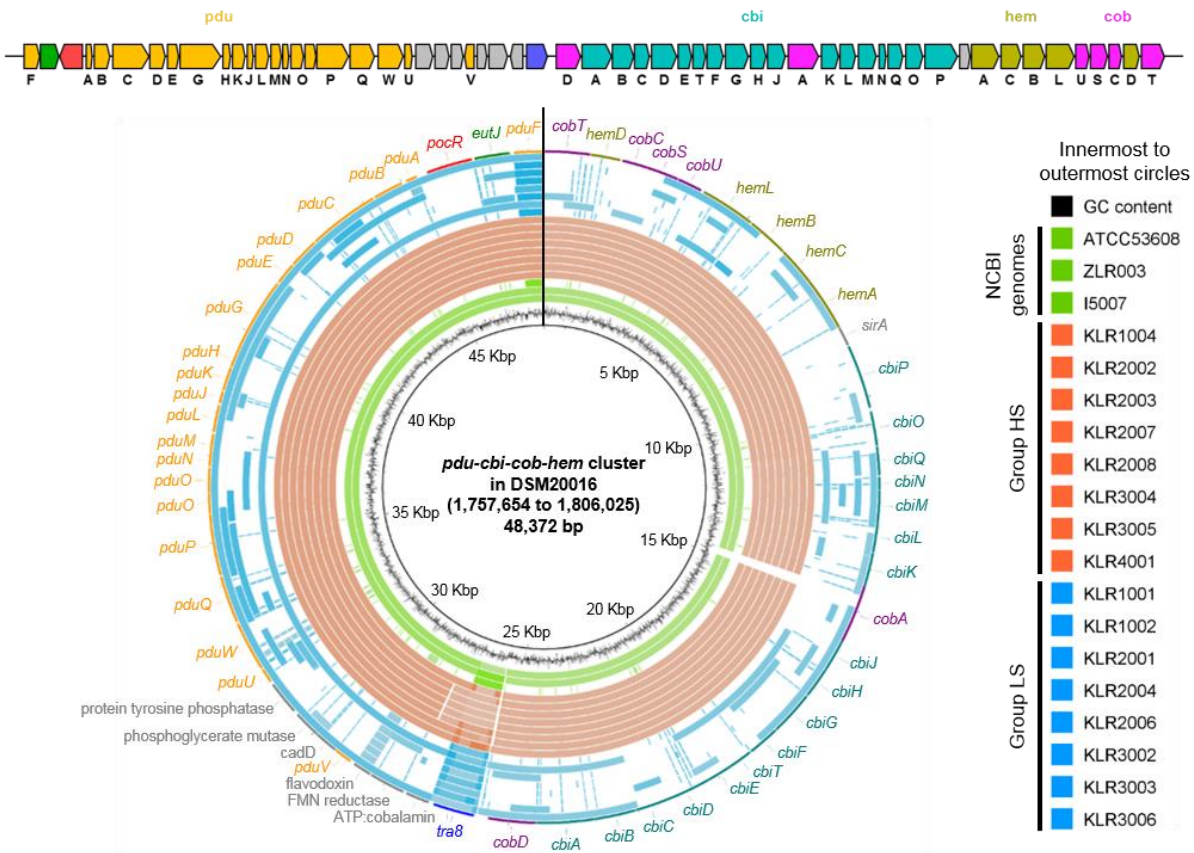


Figure 49. Circular comparison of *pdu-cbi-cob-hem* cluster of 19 porcine *L. reuteri* strains.

The genomes were aligned to the *pdu-cbi-cob-hem* gene cluster of DSM20016. The cluster of DSM20016 is shown in the upper side.

(3) HS-specific genes in *pdu-cbi-cob-hem* cluster

Among the 58 genes of *pdu-cbi-cob-hem* cluster, six genes were not present in all strains of group LS; *hemD* (locus tag LREU_RS08850 in the reference strain DSM20016), *hemB* (LREU_RS08875), *sirA* (LREU_RS08890), *cbiP* (LREU_RS08895), *cbiO* (LREU_RS08900) and *cbiD* (LREU_RS08965). These genes encode key enzymes for cobalamin biosynthesis through an anaerobic pathway. ATCC53608, ZLR003 and eight strains of group HS had the full nucleotide sequences of these genes. Two strains of group LS contain partial nucleotides of the *cbiD* (KLR1002 and KLR3003), *hemB* (KLR1002 and KLR3006), *hemD* (KLR1002 and KLR3002) genes, and strain KLR3003 in group LS contained a small fraction of the *cbiO* gene.

These genes were analyzed at the nucleotide level. The nucleotide sequence of each gene varied depending on the strain, and this result differed from that obtained by MLSA clustering. In phylogenetic analysis based on MLSA, *L. reuteri* clade VI (CF48-3A and SD2112) had the longest divergence branch from clade IV, but in the nucleotide sequences of the six genes, clade IV and VI are closely located (Figure 50). This suggests that *pdu*, *cbi*, *cob* and *hem* genes might have been transferred into the *L. reuteri* genome before host specialization occurred. Indeed, as an example, a comparison of *cbiD* nucleotide sequences among bacterial species showed that all *L. reuteri* strains are close to *L. monocytogenes* EGD-e, regardless of nucleotide polymorphisms between *L. reuteri* strains (Figure 51). Another study also demonstrated that *L. monocytogenes* and *C. difficile* are

closely related species to *L. reuteri* when amino acid sequences of CbiC were compared (Santos et al., 2008). *L. reuteri* strains may have obtained the cobalamin synthesis-associated genes from the ancestor of *L. monocytogenes* (or a closely related species), although many other species possess the genes for cobalamin and 3-HPA production and are potential gene donors. These nucleotide polymorphisms are also unrelated to the geographical origin of the strains. Taken together, these findings indicate that after *L. reuteri* acquired the *pdu-cbi-cob-hem* cluster, they evolved for adaptation to habitats such as the animal intestine while they lost some genes of the cluster.

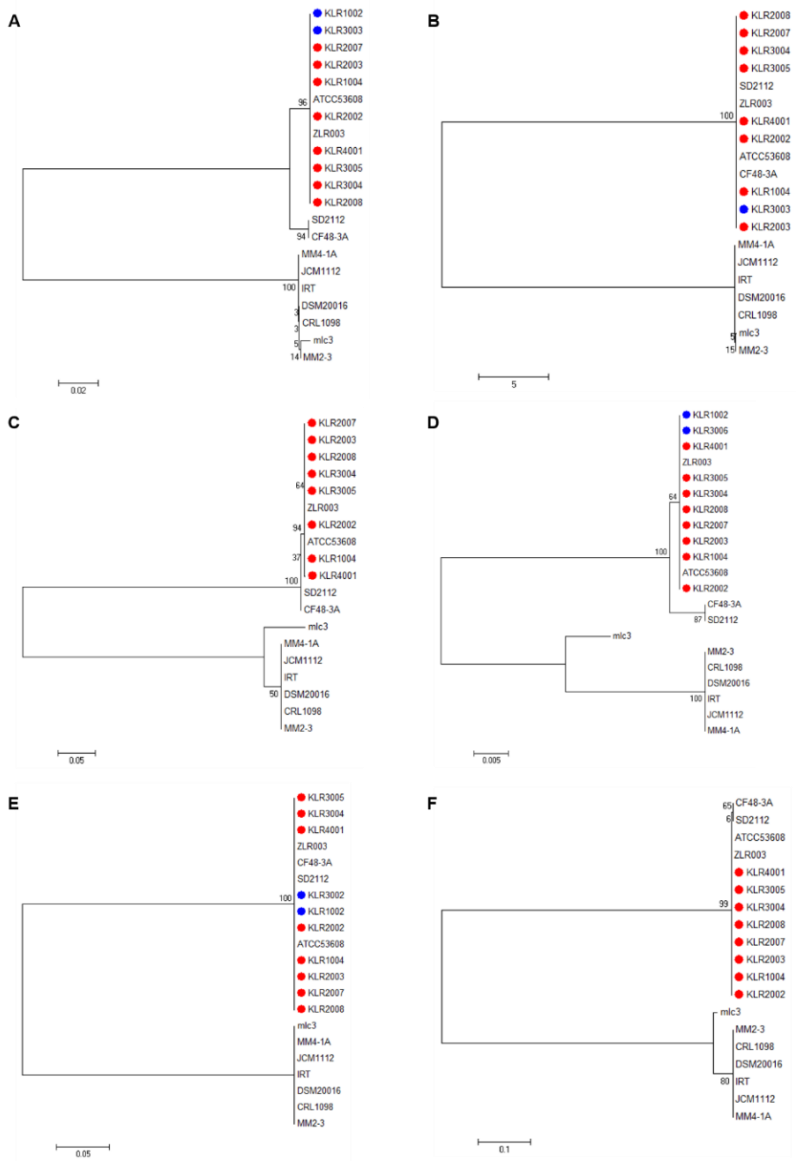


Figure 50. Phylogenetic tree of the six genes that are only identified in group HS.

(A) *cbiD*. (B) *cbiO*. (C) *cbiP*. (D) *hemB*. (E) *hemD*. (F) *sirA*. Red, group HS; blue, group LS. The partially presented genes of group LS were also used in this analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

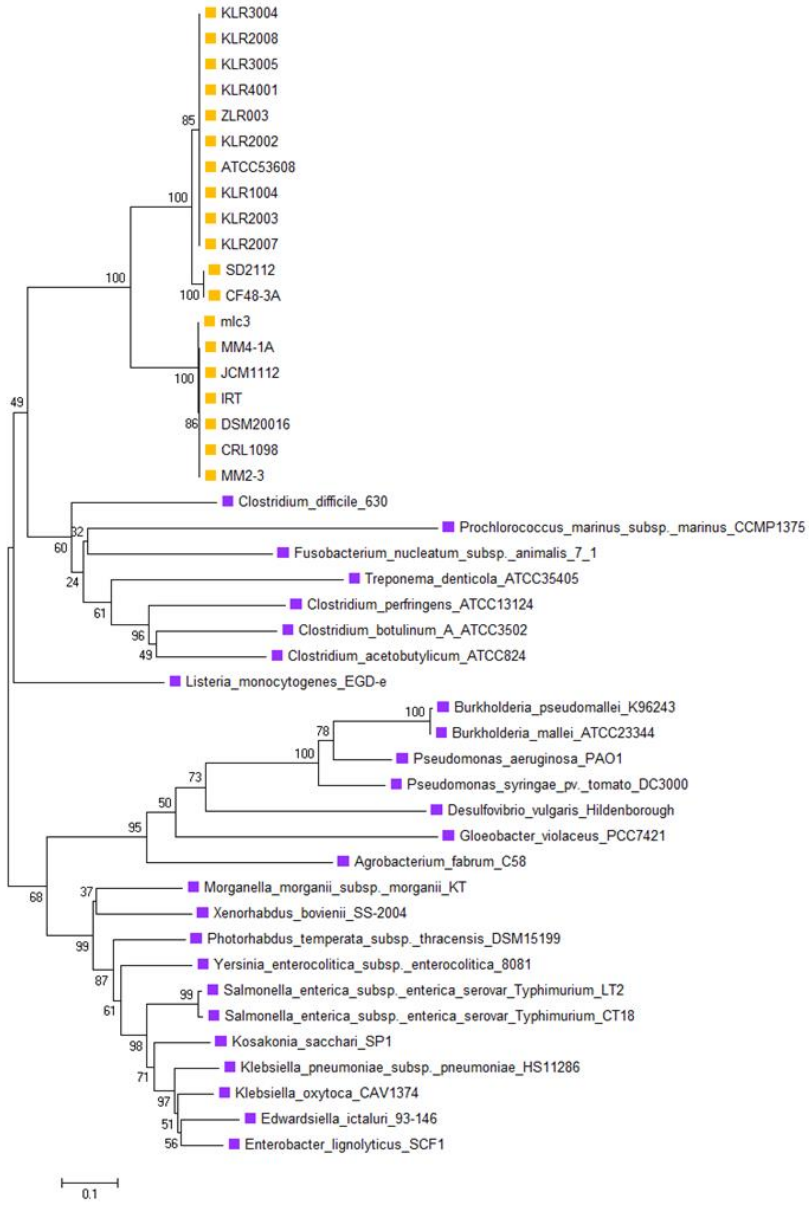


Figure 51. Comparison of nucleotide sequence of *cbiD* gene.

The optimal tree with the sum of branch length = 8.18 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Gold, *L. reuteri* strains; purple, strains of other species.

(4) Development of a selection method for antipathogenic *L. reuteri*

To confirm that these genes were present in group HS and absent from group LS, six genes were amplified by PCR. Additionally, ATCC53608 was tested at the same time for validation, because it was confirmed that this strain has antipathogenic activity (Muthukumarasamy et al., 2003) and its genomic characteristics are similar to the HS strains. The primers for PCR were designed with consideration for strain-specific polymorphic sites of the genes and avoidance of nucleotide positions of genes only partially present in some strains of group LS (Table 9). The six genes were detected in all strains of group HS but not in group LS (Figure 52A).

These genes will be used as indicator of reuterin production and genetic markers for selection of antipathogenic *L. reuteri* as a probiotic. In this aspect, a multiplex PCR method using these genes was invented. Primers for *cbiO*, *sirA*, *cbiP* and *hemB* genes were designed with considering of the strain-specific polymorphic sites of the genes, and amplicons should have different size. The primer set for each gene was tested, and the amplicon size and band intensity were confirmed in agarose gels (Figure 52B). After then, four primer sets were added to the PCR mixture for multiplex PCR. Four bands in the agarose gel for each strain which possesses complete *pdu-cbi-cob-hem* cluster were detected, and the strains belong to group LS did not show the bands (Figure 52C). This method will be useful to select probiotic *L. reuteri* strains for prevention of pathogenic infection.

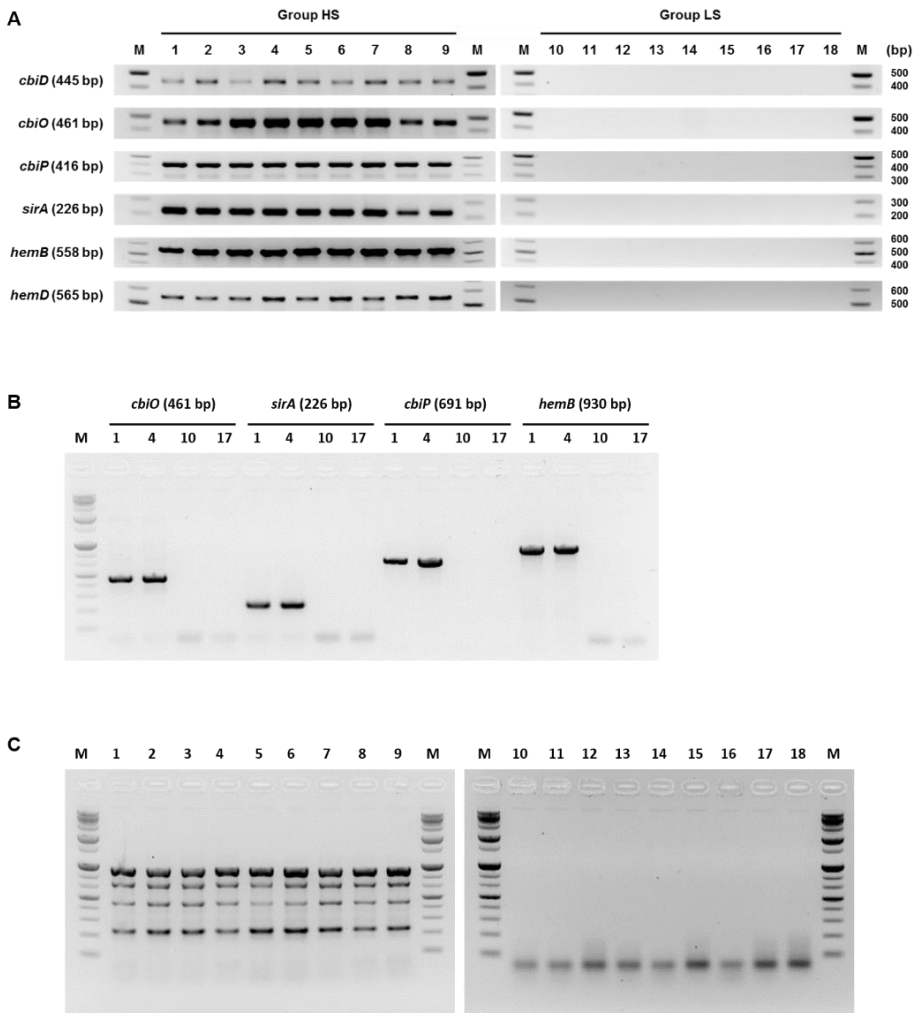


Figure 52. PCR identification of the six genes which are present only in group HS among *pdu-cbi-cob-hem* cluster.

(A) The genes existed in group HS. (B) PCR result of *cbiO*, *sirA*, *cbiP* and *hemB* for multiplex PCR. (C) Multiplex PCR with the four genes from panel B. Used primers in this figure are presented in Table 9 and Table 10. M, DNA molecular weight marker; 1, ATCC53608; 2, KLR1004; 3, KLR2002; 4, KLR2003; 5, KLR2007; 6, KLR2008; 7, KLR3004; 8, KLR3005; 9, KLR4001; 10, KLR1001; 11, KLR1002; 12, KLR2001; 13, KLR2004; 14, KLR2006; 15, KLR3002; 16, KLR3003; 17, KLR3006; 18, Negative control.

(5) Presence of citrate metabolism in some LS strains

The subsystem Citrate Metabolism, Transport, and Regulation was observed in four genomes of group LS, as indicated above (Figure 47). Several heterofermentative LAB utilize citrate for pyruvate metabolism. Citrate is transported inside the cell by a permease and converted to pyruvate by the concerted action of citrate lyase and oxaloacetate decarboxylase (Cocaign-Bousquet et al., 1996). The *citQRP* operon encodes the citrate transport system, and transcription of this operon is primarily driven by the pH-regulated P1 promoter (de Felipe et al., 1995). The transporter catalyzes uptake of divalent citrate in exchange for monovalent lactate, which results in a membrane potential of physiological polarity. Citrate is used as an energy source for bacteria with the capacity to metabolize this substrate, and it contributes to the growth and maintenance of the cell.

Although the main function of 3-HPA is the suppression of surrounding microbes, this compound also contributes to bacterial growth in glucose. 3-HPA serves as an electron acceptor in reuterin-producing *L. reuteri* strains. 3-HPA can be converted to 1,3-propanediol in a reaction catalyzed by 1,3-propanediol dehydrogenases, thereby regenerating one NAD+ molecule and retaining the redox balance during energy metabolism (Talarico et al., 1990). Addition of glycerol to a culture medium facilitated the production of reuterin, and moreover, increased the growth rate of *L. reuteri* (El-Ziney et al., 1998). Since this bacterium preferentially uses this electron acceptor for growth, pyruvate is oxidized to

acetate, synthesizing ATP, through the reaction catalyzed by acetate kinase. The strains of group LS do not convert glycerol to 3-HPA. Considering the importance of 3-HPA in *L. reuteri*, its absence from LS strains may have reduced their fitness in their primary habitat, the porcine gut. Hence, these bacteria would have evolved to survive in a different manner than the reuterin-producing strains and enhanced energy production through citrate metabolism may be helpful to their survival. Four strains of group LS acquired the genes for citrate utilization during the evolutionary process.

4. Conclusion

The *L. reuteri* strains that showed the higher (HS) or lower antibacterial activity (LS) were isolated and it was identified that between-group differences in the *pdu-cbi-cob-hem* gene cluster, which is involved in reuterin and cobalamin synthesis. Although the strains of group HS possessed all genes of this cluster, LS strains have lost many genes of the cluster. In this analysis, it was found that six genes were only identified in HS strains. As described above, probiotics and their antimicrobial activity are receiving increasing attention after the governmental ban of AGPs in the animal industry. However, a simple method for the selection of effective probiotics is needed, because screening of antipathogenic probiotics is a labor-intensive and time-consuming job. In this regard, the possible utilization of some cobalamin synthesis-associated genes as genetic markers is proposed for screening effective probiotic strains, although a more in-depth study is needed.

Furthermore, given the pan-genome of *L. reuteri*, this genetic analysis helps to characterize the ecology and evolution of *L. reuteri* with the swine host. The porcine *L. reuteri* strains evolved host-specific genes that increase fitness to their habitat. The porcine-specific genes identified are transposase and cell surface protein genes. These genes are co-localized with other transposase or cell surface protein genes, indicating that porcine-specific genes are acquired by HGT during the evolutionary process. Host-specialized cell surface proteins play a crucial role in host adaptation to their habitat, such as the epithelia of the host GIT. This

indicated that although probiotics can provide benefits to their host, they are useless if the host specialization is not considered. The identified host-specific genes may provide a basis for future studies on *L. reuteri* and other commensal bacteria.

Interestingly, the genes examined in this study are critical to the fitness of *L. reuteri* and were transferred from pathogenic bacteria. However, *L. reuteri* is GRAS and used as a probiotic bacterium. These findings suggest that *L. reuteri* might have evolved with no acquisition of pathogenic genes because the host prevents colonization by strains that exhibit pathogenicity. Additional in-depth research examining these genetic features would be worthy of investigation.

To the best of the knowledge, this report is the first to use genome sequencing to compare genomic features of animal intestinal bacterial strains producing higher versus lower levels of reuterin. Although further studies are required to determine why several genes of the *pdu-cbi-cob-hem* cluster were lost during the evolutionary process, this study provides a deeper understanding of the reuterin and cobalamin synthesis gene cluster. This study also suggests that the change in the genomic profiles may have occurred not only in *L. reuteri* but also in other bacteria that contain a similar gene cluster.

Overall Conclusion

Probiotics are regarded as AGP alternatives which have an ability to modulate the gut microbiota of animals and provide health benefits to hosts. However, unproven probiotic strains are used in the animal and functional food industry, and in this aspect, it is necessary to understand exact functions and molecular mechanisms of probiotics. Not only health benefits of probiotics to hosts, but also their host adaptation properties should be considered when development and selection of probiotic strains. Hosts and their symbionts have co-evolved with reciprocal interaction, and the symbionts were specialized to the hosts. This host-specialized microbes have host adaptation properties including mutualistic characters. Therefore, understanding direction and features of microbial evolution is important to gain the insight of probiotics and their physiological traits. Comparative genomic approach help the researchers to understand functional properties and evolution of probiotic microbes, and furthermore, ecology of microorganisms. Among the probiotic microbes, two *Lactobacillus* species that are important members in the gut microbiota and known as promising probiotic species were selected for investigation.

In study 1, pan-genome of *L. salivarius* was investigated in terms of host adaptation and the influence of the ban of AGPs. It was found that several host-specific features are associated with host adaptation features, including adhesion and colonization, nutrient utilization, and resistance against host stress.

Furthermore, these host-specific traits have a mutualistic trait being beneficial to both the hosts and the *L. salivarius* strains. The legal prohibition of AGPs changed a genome of *L. salivarius* inhabiting to the porcine intestine or the portion of the strains which possess the ability for biofilm formation in the *L. salivarius* population, with increasing biofilm formation and survival rate in competition with other persistent microbes. These features of the strains that isolated after the ban of AGPs are important as probiotics.

In study 2, host adaptation traits and antipathogenic ability of *L. reuteri* were analyzed. Most of the pig isolates-specific genes are responsible for adherence to the host habitat, indicating porcine *L. reuteri* strains have evolved with focusing on adhesion and colonization among the host adaptation traits. In the investigation of antipathogenic ability, the strains that showed higher reuterin activity had whole gene cluster for reuterin and cobalamin production, although the strains that showed the lower activity did not possess some genes of the cluster. Based on this result, a genetic marker for selection of antipathogenic *L. reuteri* strains and a method using this marker were developed.

The common and different genomic features between these two *Lactobacillus* species will be helpful to understand ecology and functional properties of lactobacilli and other probiotic bacteria. The genomes of the 35 *L. salivarius* and 42 *L. reuteri* strains used in this study were collected and compared in terms of gene functions through RAST annotation. 320 subsystems were found in the genomes of *L. salivarius* and *L. reuteri*, and 165 of 320 subsystems were shared

in all genomes of both species. Among them, 117 subsystems showed that a difference of the gene number of each subsystem is less than one (the difference = 0 in 70 subsystems, and 0 < the difference < 1 in 47 subsystems). The difference of 32 subsystems is one to two genes, and that of 16 subsystems is more than two genes. It is assumed that if the difference of the gene number in a subsystem is less, a biological role of the subsystem is more analogous in two species. An interesting feature is that they shared small portion of the total subsystems (subsystems with identical gene number is 70 of 320, 21.88%), although these species belong to the same genus. Most of shared subsystems are essential for bacterial life, such as DNA replication and repair, protein production (including chaperones), lactate fermentation, amino acid synthesis, metal ion utilization, peptidoglycan synthesis, and cytoskeleton formation. Interestingly, several traits for host adaptation were found in this analysis. All strains of the two species possess two subsystems, Galactosylceramide and Sulfatide Metabolism and Streptococcus pyogenes Recombinatorial Zone, which are related with pathogenic invasion and adhesion to hosts. It is assumed that the genes of these subsystems function to adhere to the host habitats in the non-pathogenic lactobacilli. Furthermore, some defensive mechanisms that are capable for resistance against several stress conditions were found in both *L. salivarius* and *L. reuteri*. Genes of subsystems for Housecleaning Nucleoside Triphosphate Pyrophosphatases, Biphenyl Degradation, Mercury Resistance Operon, Beta-Lactamase, and Bile Hydrolysis were identified. These genes help the bacterial survival of two species.

On the other hands, 29 subsystems were only present in one species (12 subsystems in *L. salivarius* and 17 subsystems in *L. reuteri*, Table 13). Among them, 9 subsystems are associated with carbohydrate metabolism, suggesting that this is attributed to the difference in the glycolysis and fermentation pathway; *L. salivarius* is obligately homofermentative and uses Embden-Meyerhof-Parnas pathway for glycolysis, which produces two lactic acid molecules from one glucose; *L. reuteri* is obligately heterofermentative and uses phosphoketolase pathway that converts one glucose to one lactic acid and one ethanol. This difference in utilization of a carbon source would cause the different distribution of the subsystems associated with carbohydrate metabolism. For example, a subsystem for Acetyl-CoA Fermentation to Butyrate, which is present only in *L. reuteri*, is responsible for production of SCFAs using acetyl-CoA in the phosphoketolase pathway. A subsystem for D-Gluconate and Ketogluconates Metabolism is also involved in the phosphoketolase pathway. On the other hands, subsystems for Fructose Utilization and Mannitol Utilization are necessary to produce energy in the Embden-Meyerhof-Parnas pathway of homofermentative bacteria, such as *L. salivarius*. Other subsystems including amino acid and protein metabolism and lipid metabolism were also distinguished in the two species. The common and different genetic features between *L. salivarius* and *L. reuteri* will be helpful to understand lactobacilli and probiotic bacteria.

The pan-genomic analyses of *L. salivarius* and *L. reuteri* revealed several host adaptation traits. The host-specific traits of the bacteria identified in these studies

may provide a basis for future studies of not only *L. salivarius* and *L. reuteri*, but also other symbionts. These two lactobacilli showed the mutualistic properties that are acquired during the evolutionary process. The bacterial traits for host adaptation help to select probiotic strains, and understand ecology of gut symbionts across the board. *L. salivarius* strains isolated after the AGP prohibition showed the increased biofilm formation. This result revealed that the ban of AGPs affected to the genetic materials of the bacteria or the species population, and provides the insight for probiotic selection. The comparison of higher and lower antipathogenic ability of *L. reuteri* strains showed the genetic differences in reuterin and cobalamin production of the two groups, and a selection method for probiotic *L. reuteri* was developed. These studies based on comparative genomics deepen our knowledge about evolution and ecology of gut symbionts, and provide the precise information of probiotic bacteria. Moreover, the studies can contribute to the livestock industry which is suffered from absence of effective AGP alternatives.

Table 13. Species-specific subsystems in comparison of *L. salivarius* and *L. reuteri.*

Category	Subcategory	Subsystem	Species*
Amino Acids and Derivatives	-	Creatine and Creatinine Degradation	R
	Arginine; urea cycle, polyamines	Arginine and Ornithine Degradation	R
		Arginine Deiminase Pathway	R
	Aromatic amino acids and derivatives	Phenylalanine and Tyrosine Branches from Chorismate	R
	Glutamine, glutamate, aspartate, asparagine	Glutamate dehydrogenases	S
	Proline and 4-hydroxyproline	A Hypothetical Protein Related to Proline Metabolism	R
Carbohydrates	Aminosugars	Chitin and N-acetylglucosamine utilization	S
	Central carbohydrate metabolism	Dihydroxyacetone kinases	S
		Glycolate, glyoxylate interconversions	S
	Fermentation	Acetyl-CoA fermentation to Butyrate	R
	Monosaccharides	Fructose utilization	S
		Deoxyribose and Deoxynucleoside Catabolism	R
		D-gluconate and ketogluconates metabolism	R
	Polysaccharides	Glycogen metabolism	S
	Sugar alcohols	Mannitol Utilization	S
DNA Metabolism	DNA repair	2-phosphoglycolate salvage	S
Dormancy and Sporulation	-	Sporulation Cluster	R
Fatty Acids, Lipids, and Isoprenoids	Fatty acids	Fatty acid metabolism cluster	R
	-	Polyhydroxybutyrate metabolism	R
	Triacylglycerols	Triacylglycerol metabolism	R
Miscellaneous	-	Broadly distributed proteins not in subsystems	S
		Bacillus subtilis scratch - gjo	S
Protein Metabolism	Protein degradation	Proteasome bacterial	S
	Protein processing and modification	Protein deglycation	R
Regulation and Cell signaling	-	DNA-binding regulatory proteins, strays	R
		LysR-family proteins in Salmonella enterica Typhimurium	R
Secondary Metabolism	Plant Alkaloids	Alkaloid biosynthesis from L-lysine	R
Stress Response	Oxidative stress	Cluster containing Glutathione synthetase	R
Sulfur Metabolism	Organic sulfur assimilation	Alkanesulfonates Utilization	S

* S, present in *L. salivarius*; R, present in *L. reuteri.*

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Summary in Korean

생균제는 성장촉진용 항생제의 대체제로 각광받고 있지만 축산 및 기능성 식품 분야에서는 검증되지 않은 생균제가 빈번하게 사용되고 있다. 따라서 생균제로 이용되는 미생물의 정확한 기능과 기작을 이해하는 것이 필요하며, 생균제 선발에 있어서는 숙주에게 미치는 이로운 효과뿐만 아니라 숙주 적응 능력 또한 반드시 고려되어야 한다. 숙주 특이적으로 변화된 미생물들은 진화 과정 중에 숙주 적응 능력을 얻게 되는데, 따라서 미생물의 진화 방향이나 진화의 특성을 이해하는 것이 생균제를 선발하는데 있어 중요한 요소라고 할 수 있다. 비교유전체학적 접근은 생균제 미생물의 기능적 그리고 생태적 특성을 이해하게 해주는 데에 중요한 역할을 할 수 있다. 본 연구에서는 생균제로 이용되는 미생물 중 *L. salivarius* 와 *L. reuteri* 를 선택하여 앞서 언급한 특성들을 분석하였다. 이 두 미생물 종은 동물의 장내에서 매우 중요한 구성원이며 효과적인 생균제 종으로 알려져 있지만, 이에 대한 연구는 비교적 부족한 실정이다. 대한민국의 여러 돼지 농장에서 돼지 분변을 채취하였고, 그 분변에서 634 개의 유산균주를 분리하였다. 그 중, 종 특이적 다중 중합효소연쇄반응 (multiplex PCR)을 통하여 284 개의 *L. salivarius* 균주와 104 개의 *L. reuteri* 균주를 동정하였다.

Study 1 에서는 284 개의 *L. salivarius* 균주 중에 21 개의 균주를 무작위로 선택하여 유전체 서열을 확보하였다. 이 균주들은 항생제 금지 전에 분리된 6 개의 균주 (SBP)와 항생제 금지 후에 분리된 15 개의 균주 (SAP)로 구성되어 있었다. NCBI 유전체 데이터베이스에서 확보한 인간,

돼지, 닭에서 분리된 *L. salivarius* 균주들을 포함하여, 숙주 적응에 초점을 맞춘 범유전체 (pan-genome) 분석을 실시하였다. 다중 유전자좌 서열 분석 (MLSA)을 기반으로 한 계통 분석에서 *L. salivarius* 균주들은 분리된 숙주에 따라서 묶이는 것을 확인하였다. 범유전체에 속한 4,431 개의 이종상동성 유전자 (ortholog) 중, 15 개의 숙주 특이적 유전자와 두 숙주만 보유한 16 개의 유전자를 발견하였다. 그 중 몇몇 유전자는 숙주 특이적인 세포 외부 단백질과 관련되어 있었으며, 56 개의 세포 외부 단백질 유전자와 124 개의 EPS 유전자를 분석해본 결과 마찬가지로 숙주에 따라 유전자의 분포와 특성이 다른 것을 확인할 수 있었다. 또한 몇몇 숙주 특이적 유전자들은 에너지 생산 또는 숙주로부터의 위협을 피하는 데에 역할을 하는 것을 발견하였다. 따라서, *L. salivarius* 는 숙주 환경에서 살아남기 위해 숙주 환경에 부착할 수 있는 능력 확보, 숙주 환경의 영양소 이용 능력 확보, 숙주의 위협을 피하는 능력 확보의 세 가지 방향을 가지고 진화하였다는 것을 알 수 있었다. 또한, 이러한 유전자들은 상리공생의 특성을 가지고 있는 것을 발견하였으며 이러한 특성은 숙주와 미생물의 생존력을 증가시켜주는 데에 큰 도움이 될 것으로 보인다.

또한 성장촉진용 항생제의 금지가 생균제 미생물의 유전체에 어떤 영향을 주었는지를 분석하였다. SBP 는 SAP 에 비해 유전체 크기가 더 크고 유전자 개수도 더 많았지만 항생제 저항성 유전자의 개수와 특성에는 차이가 없었다. SBP 는 L-rhamnose 와 D-tagatose 를 이용하여 에너지 생산 또는 뉴클레오타이드 (nucleotide) 합성에 기여할 수 있는 유전자들을 가지고 있었는데, 이 당들은 미생물막 (biofilm) 형성의 중요한 요소 중 하나인 EPS

합성에도 이용될 수 있다. 따라서 SBP 의 경우 위의 당들을 에너지 생산과 뉴클레오타이드 합성에 이용할 것이라 예측하였으며, 반면에 SAP 는 이러한 기능을 수행할 수 없으므로 상대적으로 미생물막을 형성하는 데에 뛰어날 것이라 추측되었다. 유전자 수준에서 분석해본 결과 SBP 와 SAP 에서 미생물막 형성에 관여하는 세포벽 단백질과 EPS 합성 유전자들의 분포 및 서열이 다름을 확인하였다. 실제로 SBP는 SAP에 비해 미생물막을 생산하는 능력이 부족함을 시험관 수준에서 관찰할 수 있었으며, SAP 의 우수한 미생물막 생산 능력은 다른 돼지 장내미생물과의 경쟁에서 살아남을 수 있는 힘을 주었다.

Study 2 에서는 *L. reuteri*의 숙주 적응과 항균 활성에 대하여 분석하였다. 돼지 분변에서 분리된 104 개의 *L. reuteri* 균주들을 대상으로 돼지 자돈에게 설사를 유발하는 두 병원균 (*E. coli* K88, *S.* Typhimurium)을 억제하는 능력을 평가하였다. 그 중 항균능력이 우수한 8 개 균주 (HS)와 저조한 8 개 균주 (LS)를 선발하여 유전체 서열을 확보하였다. 이 16 개 균주의 유전체와 더불어 NCBI 데이터베이스에 등록된 26 개의 *L. reuteri* 유전체를 함께 범유전체 분석에 사용하였다. *L. reuteri* 범유전체는 6,250 개의 이종상동성 유전자를 가지고 있었다. 우리가 분리한 16 개 균주와 NCBI 에서 얻은 돼지 유래 균주의 유전체는 모두 *L. reuteri* 계통 (clade) IV 에 속하는 것을 알 수 있었다. 이 계통 특이적 유전자는 6 개였고 그 중 두 개의 세포 표면 단백질 유전자가 포함되어 있었는데, 이는 *L. reuteri* 계통 IV 가 숙주 환경에 적응하기 위해 수평적 유전자 이동 (HGT)을 통해 얻어낸 유전자들일 가능성이 있다.

병원균에 대한 항균활성 차이를 확인하고자 HS 와 LS 의 유전체를 비교분석하였는데, reuterin 과 cobalamin 합성에 결정적인 역할을 하는 *pdu-cbi-cob-hem* 유전자군에서 큰 차이를 보였다. 이 유전자군에서 6 개의 유전자 (*cbiD*, *cbiO*, *cbiP*, *sirA*, *hemB, hemD*)는 HS 에서만 존재하며 LS 에서는 존재하지 않았다. 이 유전자들을 이용하여 항균활성 우수 *L. reuteri* 균주를 선발할 수 있는 유전자 마커 및 방법을 개발하였다.

L. salivarius 와 *L. reuteri* 의 범유전체 분석을 통해 다양한 숙주 적응과 관련된 특성들을 밝혀낼 수 있었으며, 이 중에는 숙주와 미생물의 상리공생에 관련된 유전자들이 포함되어 있었다. 이러한 점들은 숙주와 미생물간의 상호작용에 따른 진화적 변화를 이해하는 데 있어 중요한 역할을 할 것이며, 추후 두 미생물 종뿐만이 아닌 다른 공생균들에 대한 연구에도 도움을 줄 것이다. *L. salivarius* 와 *L. reuteri* 의 생균제적 특성을 비교한 연구에서는 상리공생 미생물의 또 다른 특성들을 확인할 수 있었다. 성장촉진용 항생제 금지가 *L. salivarius* 의 유전체에 미친 영향은 매우 컸으며, 항생제가 금지된 이후 격화된 장내미생물간의 경쟁에서 살아남기 위해 미생물막을 보다 많이 만들 수 있도록 진화하였다. 또한 항균활성 우수 및 저조 *L. reuteri* 균주들을 비교하였을 때에는 cobalamin 합성이 항균활성에 매우 중요한 역할을 하는 것을 확인하였고, 이를 이용하여 항균활성 우수 *L. reuteri* 균주 선발 방법을 개발할 수 있었다. 이러한 연구들은 생균제 미생물의 생균제적 특성과 생태학적 특성에 있어 깊은 이해를 돕고, 나아가서는 효과적인 가축 생균제 개발에 기여할 것이다.