

Bifidobacterium longum subsp. *infantis* as widespread bacteriocin gene clusters carrier stands out among the *Bifidobacterium*

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ABSTRACT *Bifidobacterium* is the dominant genus, particularly in the intestinal tract niche of healthy breast-fed infants, and many of these strains have been proven to elicit positive effects on infant development. In addition to its effective antimicrobial activity against detrimental microorganisms, it helps to improve the intestinal microbiota balance. The isolation and identification of bacteriocins from *Bifidobacterium* have been limited since the mid-1980s, leading to an underestimation of its ability for bacteriocin production. Here, we employed a silicon-based search strategy to mine 354 putative bacteriocin gene clusters (BGCs), most of which have never been reported, from the genomes of 759 *Bifidobacterium* strains distributed across 9 species. Consistent with previous reports, most *Bifidobacterium* strains did not carry or carry only a single BGC; however, *Bifidobacterium longum* subsp. *infantis*, in contrast to other *Bifidobacterium* species, carried numerous BGCs, including lanthipeptides, lasso peptides, thiopeptides, and class IId bacteriocins. The antimicrobial activity of the crude bacteriocins and transcription analysis confirmed its potential for bacteriocin biosynthesis. Additionally, we investigated the association of bacteriocins with the phylogenetic positions of their homologs from other genera and niches. In conclusion, this study re-examines a few *Bifidobacterium* species traditionally regarded as a poor source of bacteriocins. These bacteriocin genes impart a competitive advantage to *Bifidobacterium* in colonizing the infant intestinal tract.

IMPORTANCE Development of the human gut microbiota commences from birth, with bifidobacteria being among the first colonizers of the newborn intestinal tract and dominating it for a considerable period. To date, the genetic basis for the successful adaptation of bifidobacteria to this particular niche remains unclear since studies have mainly focused on glycoside hydrolase and adhesion-related genes. Bacteriocins are competitive factors that help producers maintain colonization advantages without destroying the niche balance; however, they have rarely been reported in *Bifidobacterium*. The advancement in sequencing methods and bacteriocin databases enables the use of a silicon-based search strategy for the comprehensive and rapid re-evaluation of the bacteriocin distribution of *Bifidobacterium*. Our study revealed that *B. infantis* carries abundant bacteriocin biosynthetic gene clusters for the first time, presenting new evidence regarding the competitive interactions of *Bifidobacterium* in the infant intestinal tract.

KEYWORDS genome mining, bacteriocin gene clusters, *Bifidobacterium*, *B. longum* subsp. *infantis*, antimicrobial activity, RT-qPCR, niche competition

Bacteriocins are antimicrobial peptides synthesized by bacterial ribosomes that can inhibit the growth of other related or unrelated microorganisms (1). Bacteriocins

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are widespread across the human intestinal tract and are crucial in determining the composition of gut microbiota (2, 3). They help producers compete in their specific ecological niches, maintain a balance in microbial populations (4, 5), and actively participate in host defense against potential gastrointestinal pathogens (6). Many bacteriocins have been isolated and identified from the gut microbiota and are roughly divided into three classes based on molecular weight, post-translational modification, chemical structure, thermal stability, and other properties (3, 7). Class I bacteriocins are post-translationally modified peptides (RiPPs), which are further subdivided into lanthipeptides, lasso peptides, sactipeptides, autoinducing peptides (AIPs), thiopeptides, and other subgroups according to the comprehensive nomenclature of RiPPs (8). Lanthipeptides are characterized by the β -thioether cross-linked bis-amino acids lanthionine (Lan) and methyllanthionine (MeLan). Class I lanthipeptides are characterized by two proteins that individually catalyze dehydration (LanB) and cyclization (LanC). Class II lanthipeptides biosynthesis involves a single protein with both dehydration and cyclization domains (LanM). Class III and class IV lanthipeptides are characterized by distinct phosphorylation-mediated dehydration processes (9). Lasso peptides are named for their topology resembling a threaded lasso or a slipknot, rendering them resistant to heat, chemicals, and proteases (10). Sactipeptides are distinguished by radical S-adenosylmethionine (rSAM) enzymes that form intramolecular thioether linkage between a donor Cys sulfur and the alpha carbon of another acceptor amino acid (11). AIPs are peptide signals modulating the expression of virulence factors via the Agr quorum-sensing system, which comprises response regulator AgrA, histidine kinase AgrC, and protease AgrB that cleaves AgrD (precursor peptide) (12). Thiopeptides are highly modified macrocyclic peptides with a six-membered heterocycle grafted on the peptidic backbone being central to pyridine/piperidine/dehydropiperidine, and the enzymes that are necessary for primary macrocyclization, such as YcaO, ThiF, dehydrogenase, dehydratase, and macrocyclase, are generally conserved. Small (<10 kDa), heat-stable class II bacteriocins are subdivided into class IIa peptides (containing the YGNGV motif), class IIb peptides (two-peptide bacteriocins), class IIc peptides (circular bacteriocins), and class IId peptides (unmodified, linear, non-pediocin-like, and single-peptide bacteriocins) based on their structure and function (3). Class III bacteriocins are large (>30 kDa), heat-labile proteins that have not been extensively characterized (3).

Bifidobacterium is among the first bacteria that colonize and inhabit the human intestinal tract for life. Certain species, such as *B. longum* subsp. *infantis* (*B. infantis*) and *Bifidobacterium breve*, are more common in the infant gut microbiome. Other species co-exist in the intestinal tract of infants and adults, including *B. longum* subsp. *longum* (*B. longum*), *Bifidobacterium adolescentis*, and *Bifidobacterium pseudocatenulatum* (13). *Bifidobacterium* is the dominant genus in the intestinal tract of healthy breast-fed infants, and its relative abundance appears to be negatively correlated with several disease states (14). Members of the *Bifidobacterium* genus are widely recognized as beneficial health-promoting bacteria due to their extensive prebiotic functions. However, the bacteriocin-producing capability of bifidobacteria has been underestimated despite their inhibition of detrimental microbial growth (15). Typically, their antibacterial activity is attributed to the inhibition of organic acids and a decrease in pH (16). Compared to the extensively reported bacteriocin-producing lactic acid bacteria (LAB) (17), such as *Lactobacillus* and *Enterococcus*, only a few strains of bacteriocin-producing *Bifidobacterium* have been discovered, with scarce information on the genes or structure of the bacteriocins (18). The above mining of bacteriocin from bifidobacteria is based on antimicrobial phenotype to screen, which is labor- and time-intensive.

With the rapid advances in sequencing technologies, numerous bacteriocins have been identified and characterized by laborious screening methods and biochemical characterization; *in silico* screening strategy based on genomics and bioinformatics has become a more efficient means of bacteriocin mining. Genome mining tools, such as the bacteriocin mining tool BAGEL (19) and the secondary metabolite mining tool antiSMASH (20), exploit the conserved domains of genetic elements in bacteriocin gene

clusters (BGCs), enabling the easy identification of new compounds in microorganisms (21–23). Despite these advancements, the BGCs in the *Bifidobacterium* genus were rarely mined in a recent study (24), possibly due to the lack of sufficiently comprehensive data for analysis. Equipped with the available genome data of the *Bifidobacterium* genus, we addressed these shortcomings by evaluating the bacteriocin biosynthesis potential of the *Bifidobacterium* genus. Through genome mining, we characterized valuable phenotypic traits associated with antimicrobial activity and explored the potential associations among *Bifidobacterium*, bacteriocins, and niches.

RESULTS

Most *Bifidobacterium* species are poor sources of bacteriocins

We identified 354 putative BGCs from 133 *Bifidobacterium* strains (Table S3). Based on the gene organization and homology of their structural and biosynthetic genes, these BGCs were classified into three classes harboring eight subgroups, namely, lanthipeptides, lasso peptides, thiopeptides, sactipeptides, AIPs, class IIa bacteriocins, class IIb bacteriocins, and class III bacteriocins (Fig. 1). Gene clusters with the same structural composition and order under a subgroup of BGCs were grouped into a “type.” The situation of each *Bifidobacterium* species carrying BGCs was diverse. No BGC was found in *B. adolescentis*, and only one *Bifidobacterium animalis* strain carried BGCs among strains of the species. Three of the six *Bifidobacterium angulatum* strains possessed one type of class II lanthipeptide gene cluster, and 15 *Bifidobacterium dentium* strains possessed one type of class II BGC. A total of 48 *B. pseudocatenulatum* strains harbored three types of BGC, primarily (~90%) concentrated in the undetermined class IV lanthipeptide gene cluster. Thirty-eight BGCs encoding lanthipeptides, lasso peptides, AIPs, and class II and III bacteriocins were mined from 12 *B. breve* strains.

Abundant and diverse BGCs were mined in *B. infantis*

In contrast to the aforementioned *Bifidobacterium* species exhibiting a low BGC distribution frequency, *B. infantis* has great potential to produce a wide variety of bacteriocins. A total of 36 *B. infantis* isolates harbored five subgroups of 220 BGCs, and the occurrence of putative BGCs was 100%. Moreover, except class II and III bacteriocins found in two *B. breve* strains and three AIPs found in one *B. breve* strain and one *B. animalis* strain, *B. infantis* harbored all the BGCs found in other *Bifidobacterium* species. We constructed the phylogenetic tree of homologous sequences of various bacteriocins and found that most bacteriocins from *B. infantis* were native and were only distributed in the branches of *Bifidobacterium*. As an additional note, we tested four other strains (CECT7210, 157F, JCM11660, and KCTC5934) marked as *B. infantis* in NCBI; the results revealed that they did not harbor any BGC. The average nucleotide identity (ANI) analysis found that they were clustered with *B. longum* subsp. *longum* (Fig. S2). Therefore, we did not include them in our study.

The following sections describe the characteristics of various BGCs classified by bacteriocin groups in *B. infantis* in detail. The precursor peptide sequences carried by other *Bifidobacterium* species are also displayed.

Lanthipeptides

In this study, 104 putative lanthipeptide gene clusters were discovered in *B. infantis*, which was also the largest subgroup. Among these, two types of class I lanthipeptide gene clusters were distributed across the genomes of the 15 *B. infantis* strains. According to the results of basic local alignment search tool (BLAST) in the nonredundant (nr) protein database, all annotated precursor peptides of class I lanthipeptides belonged to the gallidermin/nisin family lantibiotics (Fig. 2Ac). Five different precursor peptide sequences were obtained from the first gene cluster and were found to cluster with the other lantibiotics from *Bifidobacterium* on the phylogenetic tree differing only at a few amino acid positions (Fig. 2Ab). Alignment of the precursor peptides with identified

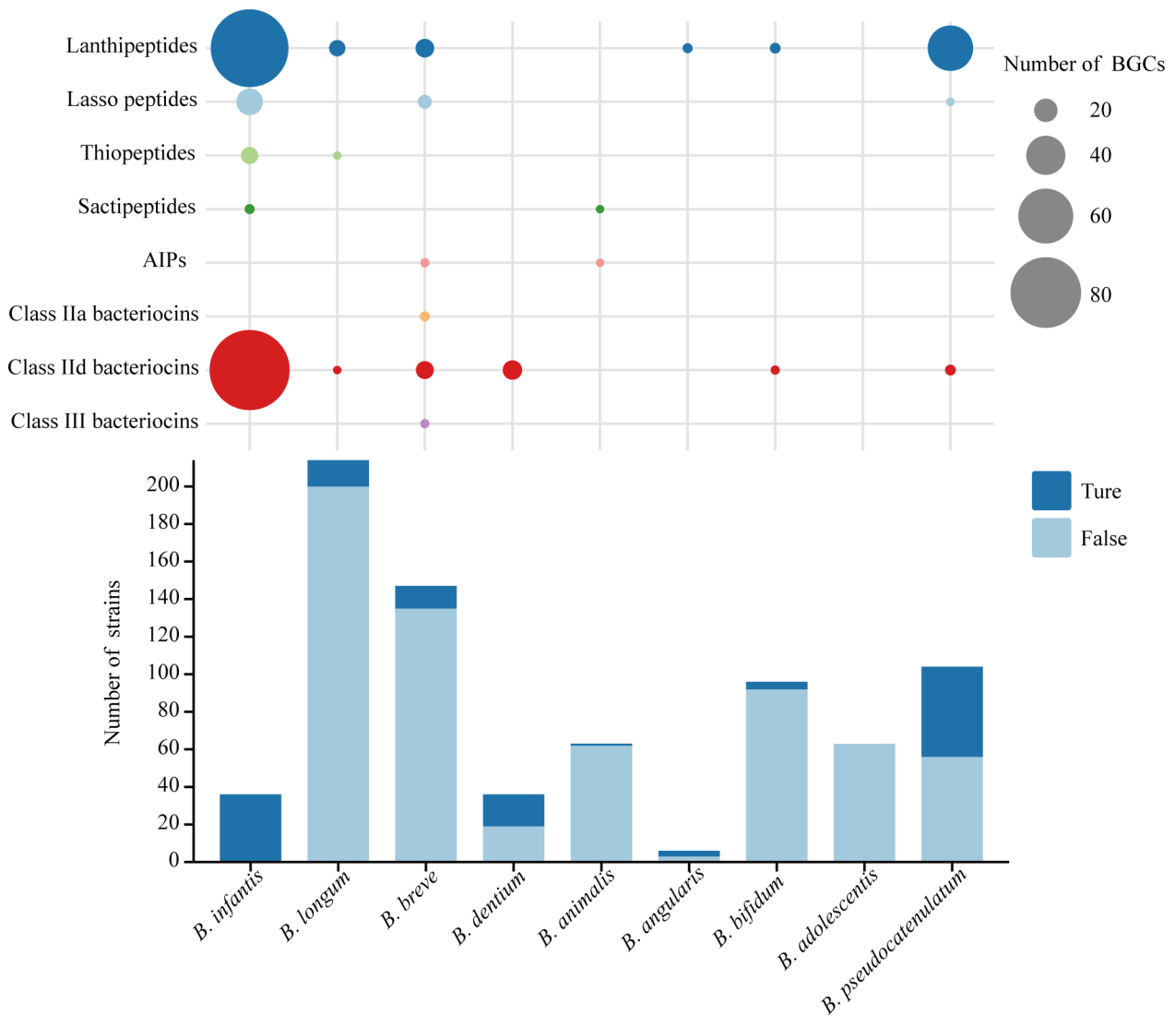
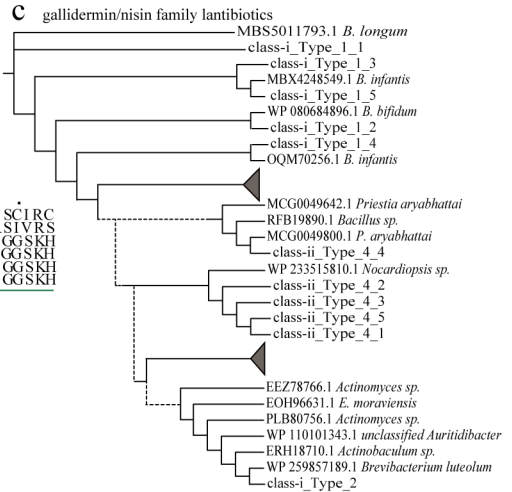
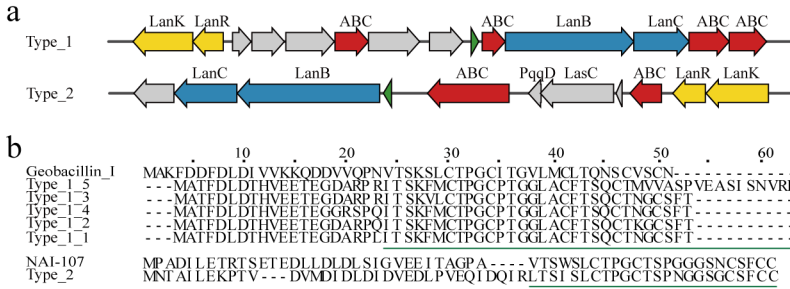


FIG 1 BGCs distribution of the *Bifidobacterium*. The bar plot shows the number of *Bifidobacterium* strains with/without putative BGCs; the bubble plot shows the number of different groups of BGCs carried by the *Bifidobacterium* species.

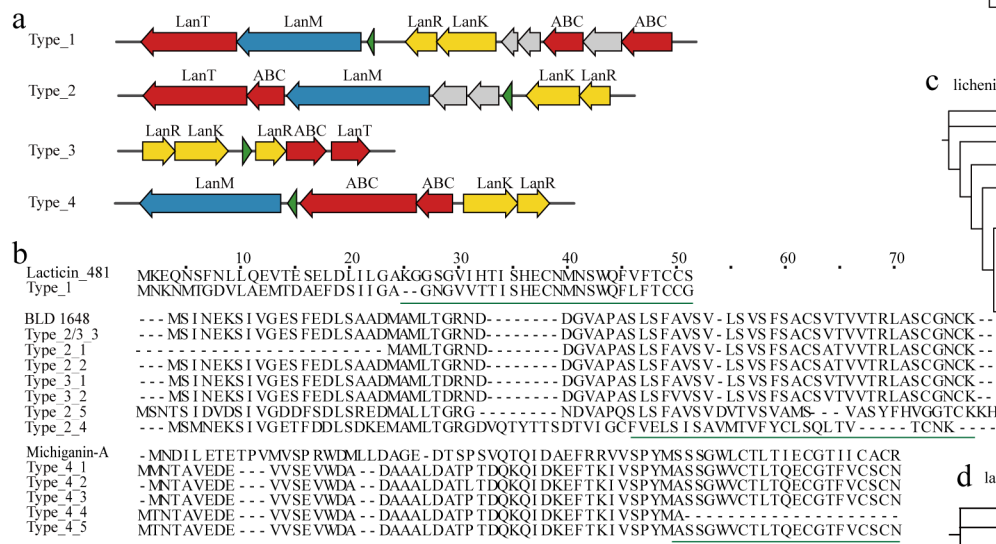
bacteriocins revealed higher similarity with the geobacillin I sequence from *Geobacillus thermodenitrificans* (25). Three *B. infantis* (FHuNCS6M8, FJND16M4, and FLNSY3M1) and two *Bifidobacterium bifidum* (FGZ3I2M1 and FGZ6I2M1) strains had class I lanthipeptide gene clusters similar to the first type; however, they lacked precursor peptide genes. After excluding the sequencing factor via reverse transcription-quantitative PCR (RT-qPCR) analysis, we assumed that the precursor genes of these strains could have been lost due to genetic drift; therefore, these strains were excluded. Similarly, two *B. dentium* strains (FNXHL9M3 and FNXHL9M2) were disregarded due to the absence of precursor genes. In the second cluster, the lanthipeptide gene cluster was adjacent to the lasso peptide gene cluster; hence, we suspect that they share the two-component regulatory system genes, LanR and LanK. One precursor peptide was obtained from the second gene cluster, which did not cluster with the specific genus. Its core peptide sequence shared 83.3% identity with the NAI-107 (also known as microbisporicin or 107,891) produced by *Microbispora* sp. (26, 27).

We identified four types of class II lanthipeptide gene clusters from 29 *B. infantis* strains (Fig. 2B). The first type was homologous to the class II lanthipeptide lacticin 481 (also known as lactococcin DR) gene cluster of *Lactococcus lactis* (28). The second cluster

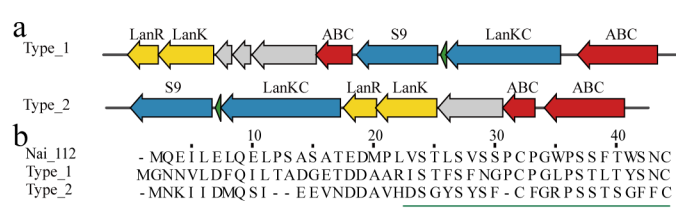
A Class I Lanthipeptides



B Class II Lanthipeptides



C Class III Lanthipeptides



D Class IV Lanthipeptides

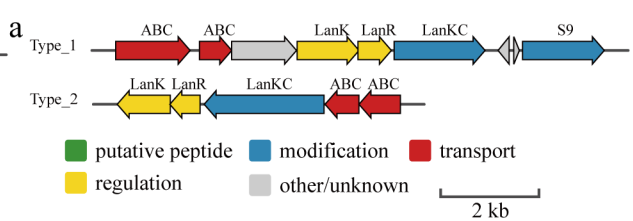


FIG 2 BGC mining results of class I-IV lanthipeptides (A-D) containing bacteriocin biosynthetic gene clusters (A), multiple sequence alignment (MSA) results of putative lantibiotic prepeptides with known bacteriocin (B), and the phylogenetic tree of homologous sequences in the NCBI (C and D). The green underline indicates the core peptide sequence in the MSAs.

had been reported to encode BLD 1648 (based on the BAGEL4 database) in *B. longum* DJO10A (2, 29). The third cluster, mined using BAGEL4, encoded a similar precursor peptide as the second cluster but lacked the important modification gene *lanM*. Although nearly half of these strains encoded other class II lanthipeptides, we could not determine the influence of the absence of *lanM* in the gene cluster on the normal activity of the lantibiotic. BLAST results identified that these compounds belong to lichenicidin A2 family type 2 lantibiotics. The fourth type was found exclusively in *B. infantis* and shared homology with five similar precursor peptides annotated as gallidermin/nisin family lantibiotics, based on BLAST results. When compared with known bacteriocins in the BAGEL4 database, they exhibited the highest similarity to michiganin-A, obtained

from *Clavibacter michiganensis* subsp. *michiganensis* (30). *B. infantis* strain M203F0227 lacked this precursor gene by annotation; however, we confirmed its presence by RT-qPCR.

Two types of class III lanthipeptide gene clusters were distributed across the genomes of 20 *B. infantis* strains, as well as 5 *B. pseudocatenulatum* strains, 1 *B. brevis* strain, and 1 *B. bifidum* strain (Fig. 2C). The precursor peptide from the first gene cluster was homologous to the class III lanthipeptide NAI-112 produced by *Actinoplanes* spp. when aligned against the sequences in the BAGEL4 database (31, 32). No known bacteriocins homologous to the second precursor peptide were identified, suggesting it may be a novel class III lanthipeptide. Additionally, we found two types of class IV lanthipeptide gene clusters, the first was identified in 3 *B. infantis*, and the second was identified in 46 *B. pseudocatenulatum* strains (Fig. 2D). We were unable to identify the potential precursor peptides using BLAST.

Lasso peptides

A phylogenetic tree was constructed for the lasso RiPP family leader peptide-containing protein sequences from NCBI (<https://www.ncbi.nlm.nih.gov/>), and the three precursor peptides were clustered with other lasso peptides from *Bifidobacterium* (Fig. 3Ad). The identified lasso peptides exhibited higher similarities to the propeptin sequence from *Microbispora* sp. SNA-115 (33, 34). We predicted the cyclization structure of core peptides based on the structure of propeptin (Fig. 3Ac).

Thiopeptides

Over 100 thiopeptides have been isolated and characterized heretofore; however, none have been isolated from *Bifidobacterium* before this study (35). We found one type of putative thiopeptide BGC, which most likely encodes a series D thiopeptide in 12 *B. infantis* strains and 1 *B. longum* strain (Fig. 3B). Although, none of the thiopeptides in the bacteriocin databases were found to be similar to the precursor peptides in this *Bifidobacterium* strain, we did identify a precursor peptide and a thiopeptide from *Cutibacterium acnes*, called cutimycin, located on a subunit in the phylogenetic tree of the thiazolylpeptide-type bacteriocin sequences (36).

Sactipeptides

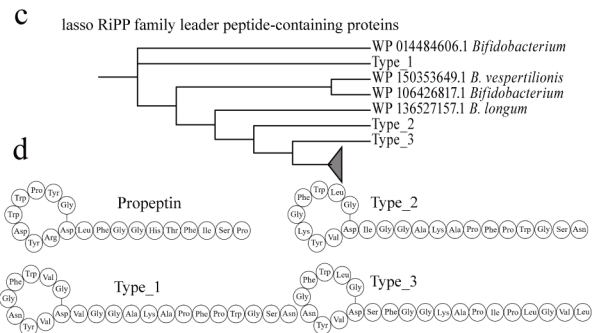
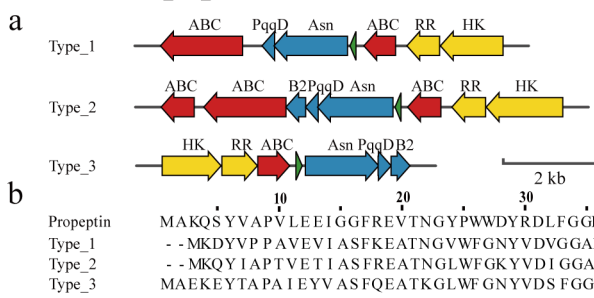
We identified two types of putative sactipeptide gene clusters in the genomes of three *B. infantis* strains and one *B. animalis* strain; however, no precursor peptides were predicted (Fig. 3C). Unlike the genomes of the three *B. infantis* strains, *B. animalis* FJSNJLH3M11 genomes encoded histidine kinase and response regulators besides encoding rSAM enzymes and ABC transporter. This is the only reported putative sactipeptide gene cluster from *Bifidobacterium*, although this finding requires further investigation.

Class IId bacteriocins

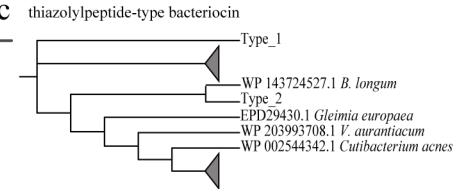
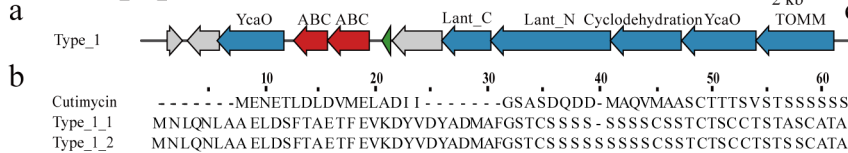
Lactococcin 972 (Lcn972) is a member of the class IId bacteriocins (37). We found one or two gene clusters encoding Lcn972 analogs in all 36 *B. infantis* strains. This type of gene cluster encodes a precursor peptide, an ABC transporter protein, and a two-component regulatory system, portraying a simple genetic organization. The precursor peptides were clustered in the phylogenetic tree of Lcn972 together with sequences from other *B. longum* strains (Fig. 3Dc). The second type of precursor peptide cluster also shared homology with Lmo2776, a bacteriocin produced by *L. monocytogenes* (38).

Propionicin SM1-like genes were mined using BAGEL4 in 59 genomes distributed among 30 *B. infantis*, 15 *B. dentium*, 8 *B. brevis*, 4 *B. pseudocatenulatum*, and 2 *B. bifidum* strains (39). Some gene clusters included only precursor peptides, without ABC transporter proteins (Fig. 3E). The overall similarity of amino acid sequences between these precursor peptides and propionicin SM1 was 26.1%–50.5%, although they were annotated as DUF2599 domain-containing proteins in the nr protein database.

A Lasso peptides



B Thiopeptides



C Sactipeptides



D Class IId bacteriocins

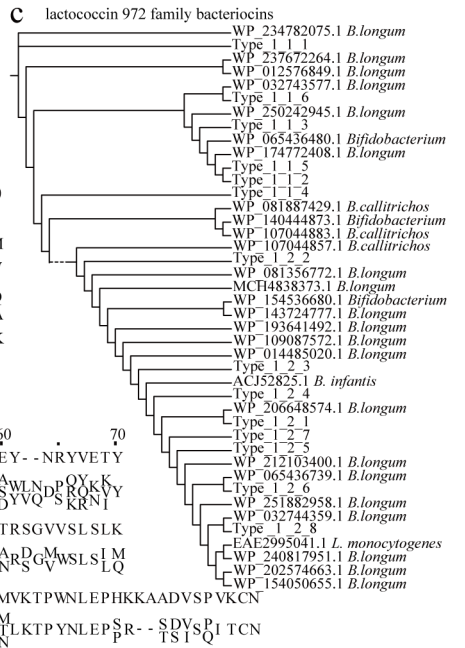
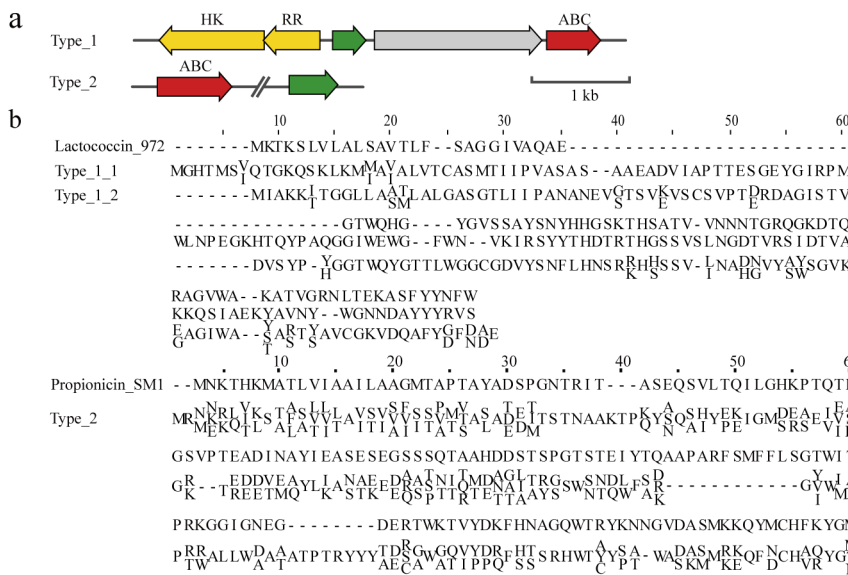


FIG 3 BGC mining results of lasso peptides (A), thiopeptides (B), sactipeptides(C), and class IId bacteriocins (D) containing bacteriocin biosynthetic gene clusters (A), multiple sequence alignments (MSAs) of putative precursor peptides with known bacteriocins (B), the phylogenetic tree of homologous sequences in the NCBI (C), and predicted structures based on identified bacteriocins (D). The green underline indicates the core peptide sequence in the MSAs. Considering that multiple sequences are only different at several amino acid sites, MSA uses the consensus results to show in D.

BGC horizontal transfer exists in *B. breve* and *B. animalis* strains

Three bacteriocins previously associated with other species were identified in *B. breve* and *B. animalis* strains. A core AIP from *Staphylococcus epidermidis* (40) was identified in *B. animalis* FJSNJLH3M1 (Fig. 4A type_1); additionally, EntL50A and EntL50B from *Enterococcus faecium* L50 (41) were identified in *B. breve* FSHMX2M1 (Fig. 4B class IId type_3). Additionally, some putative bacteriocins were identified in the above strains and

S. epidermidis, the AIP structure of *B. animalis* was shown in Fig. 4Ac (40). The structure of the other two AIPs from *B. breve* still needed experimental verification.

Seven putative bacteriocins encoding Class II and Class III bacteriocins were identified in five gene clusters of FSHMX2M1 (Fig. 4B). The precursor peptides encoded by the first and third gene cluster were similar to the class IIa bacteriocin enterocin P produced by *E. faecium* (48.3% and 56.3%, respectively) (42). Two putative bacteriocins were identified in the second cluster. One of the bacteriocins, located upstream, had 94.8% sequence similarity with the class IIa bacteriocin mundticin ATO6 produced by *Enterococcus mundtii* ATO6 (43). The other bacteriocin, located downstream, showed 29.8% sequence similarity with enterolysin A. Additionally, the bacteriocin encoded in the fourth gene cluster was similar to enterolysin A with 20.20% similarity (44, 45). The fifth gene cluster carried bacteriocins consistent with class IIb bacteriocin Enterocin L50 (46).

Two class II bacteriocin gene clusters were identified in *B. brevis* M207F01M63 (Fig. 4C). The first precursor peptide showed 60.8% sequence similarity to the class IIc bacteriocin Bovicin 255 produced by *Streptococcus gallolyticus* LRC0255 (47). This peptide and *Streptococcus*-derived peptides were clustered in the same cluster on the phylogenetic tree of the garvicin Q family class II bacteriocin. The second gene cluster carried two hypothetical class II bacteriocin genes, which were located downstream of two ABC transporter genes and showed sequence similarity to thermophilin A (68.2%) and BlpI (60.8%) (48, 49). The first precursor peptide clustered with a peptide from *Streptococcus* in the phylogenetic tree of Blp family class II bacteriocins. The second precursor peptide was grouped with peptides from *Streptococcus* in the phylogenetic tree of the lactobin A/cerein 7B family.

***B. infantis* strains and their crude bacteriocins inhibit the growth of common competitive bacteria in the infant intestinal tract**

Owing to the abundance of bacteriocin synthesis genes harbored by *B. infantis*, we tested the antimicrobial activity of 29 *B. infantis* strains against 9 bacteria that were common in infant intestines. As shown in Table 1, all 29 *B. infantis* strains exhibited broad antimicrobial activity against both Gram-negative bacteria and Gram-positive bacteria, except for the *Lactobacillus*. During testing, all crude bacteriocins lost their activity against Gram-negative bacteria (Table 2). Most crude bacteriocins showed strong inhibitory activity against *E. faecalis* and *L. monocytogenes*. Considering that each *B. infantis* strain produces several bacteriocins and other antimicrobial substances may also be present in the crude bacteriocins, the target bacteria of specific bacteriocin need to be validated later after isolation and purification.

The levels of BGCs transcription varied among the confirmed clusters

All confirmed BGCs were transcribed in the deMan Rogosa Sharpe (MRS) liquid medium at different levels (Fig. S1). Among the 29 *Bifidobacterium* strains, the genes with the least difference in transcription levels were *lan-i-T_2*, *lan-iii-T_1*, *lan-iii-T_2*, *lan-ii-T_1*, and *lasA*, whereas the transcription levels of *lan-ii-T_4*, *lcn972-T_1_1*, and *lcn972-T_1_2* were quite different. During the analysis, we found that many precursor peptide genes had low transcription intensity in the liquid medium, which complicated the screening of bacteriocin-producing *Bifidobacterium* strains using the fermentation supernatant and purification of bacteriocin (50). Using previously mentioned primers, we excluded a few BGCs that lacked the precursor gene and confirmed the BGCs whose precursor genes were disregarded due to sequencing/assembly reasons. These primers may facilitate the efficient screening of *Bifidobacterium* strains carrying bacteriocin synthesis genes.

DISCUSSION

The first putative *Bifidobacterium*-associated bacteriocin was bifidin, isolated from *B. bifidum* NCDC 1452 in the mid-1980s (51). However, the research progress on bacteriocins involving *Bifidobacterium* has been slow since then. To date, only nine putative *Bifidobacterium*-associated bacteriocins have been identified, one in *B. lactis* Bb-12 (52),

TABLE 1 The antimicrobial activity of *B. infantis* strains^a

Zone size	<i>E. faecalis</i>	<i>S. aureus</i>	<i>B. adolescentis</i>	<i>B. bifidum</i>	<i>L. plantarum</i>	<i>L. rhamnosus</i>	<i>L. monocytogenes</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>S. enterica</i>
FGZ19I2M3	+	+	+	+	-	-	++	+	++	++
FGZ23I1M7	++	++	++	++	-	-	+++	+	++	+++
FHeNJZ3M1	++	++	+	++	-	-	+++	++	+++	+++
FHNFQ45M2	+	+	++	+++	-	-	+++	+	+++	+++
FHuNC6M8	++	++	+	++	-	-	+++	++	+++	+++
FJND2M2	+	+	+	+++	-	-	+++	+	+++	+++
FJND16M4	++	++	++	++	-	-	+++	++	+++	+++
FJSYZ1M3	++	++	+	++	-	-	+++	+	+++	+++
HeNJZ8M1	+++	++	++	++	-	-	+++	++	+++	+++
JSSZ7M7	++	++	+	++	-	-	+++	++	+++	+++
JSWX3M1	++	++	++	+++	-	-	+++	++	+++	+++
JSWX6M2	+	+	+	++	-	-	++	+	+++	++
JSWX25_6	+	+	+	+	-	-	++	+	+++	+++
JSWX25M6	+	+	+	+++	-	-	+++	++	+++	+++
SDZC2M4	+	+	+	++	-	-	+++	+	+++	+++
FGZ17I1M1	++	++	+	++	-	-	+++	+	+++	+++
FGZ19I1M3	+	+	+	++	-	-	++	+	+++	+++
FGZ23I1M2	+	+	+	++	-	-	++	+	+++	+++
FHeJZ44M8	++	++	++	++	-	-	+++	++	+++	+++
M203F0227	+	+	+	++	-	-	++	++	+++	+++
FBJTZ1M6	++	++	++	++	-	-	+++	+	+++	++
FHBSJZ9M1	++	++	++	+	-	-	+++	+	++	+
FHBSJZ50M1	++	++	++	++	-	-	+++	++	+++	+
FHeBSJZ1M1	++	++	++	++	-	-	+++	+	+++	+
FHNFQ4M11	++	++	++	++	-	-	+++	+	++	+
FJSWXI2MIM1	++	++	++	++	-	-	+++	++	+++	+
FJSWXI4MIM1	+	++	++	+	-	-	++	+	++	+
FLNSY3M1	++	++	++	+	-	-	+++	++	+++	+
M207F01M51	++	++	++	+	-	-	+++	+	++	+

^aZone size = diameter of the zone - diameter of the spot in millimeters. - means no antimicrobial activity; + means 0-10 mm; ++ means 10-20 mm; +++ means >20 mm.

two in *B. bifidum* (53, 54), three in *B. longum* (29, 55), one each in *B. thermophilum* RBL67 (56), *B. animalis* BB04 (57), and *B. infantis* BCRC14602 strains (51); however, most of these bacteriocins have not been strictly characterized or sequenced. Bifidocin B, bifidin I, and BLD 1648 are the only *Bifidobacterium*-associated bacteriocins whose sequences have been partially elucidated. The mining of the above bacteriocin mainly depends on the inhibitory effects exerted by the producer on the indicator bacteria and is evaluated using agar plates or direct tests. These *in vitro* methods can be used to discover new bacteriocins and evaluate their diverse antimicrobial abilities. However, the antimicrobial ability detected by these methods is affected by nutritional and biological factors, such as medium composition, stress conditions, and the presence of target bacteria, which may result in false-negative results (18). Numerous bacterial whole-genome sequence data and BGC mining tools have made the identification of hypothetical BGCs using *in silico* analysis efficient and convenient (21, 22). In this study, we found that the proportion of BGCs harbored by each of *Bifidobacterium* species was either low or that of a single type, except for *B. infantis*, explaining the scarce detection of bifidobacterial bacteriocins previously (18). *B. infantis* stands out as a potential bacteriocin producer and harbors abundant BGCs that can serve as its distinct species marker.

In certain cases, complete gene clusters lacking evident bacteriocin precursor genes were identified, which predominantly belonged to class IV lanthipeptides and sactipeptides. Excluding low-quality genome assembly, the reasons for "unidentified" are as follows: (i) the precursor peptide is encoded elsewhere in the genome, (ii) the precursor gene is lost due to genetic drift, (iii) rSAM is not involved in sactipeptide biosynthesis

TABLE 2 The antimicrobial activity of crude bacteriocins from *B. infantis* strains^a

Zone size	<i>E. faecalis</i>	<i>S. aureus</i>	<i>B. adolescentis</i>	<i>B. bifidum</i>	<i>L. plantarum</i>	<i>L. rhamnosus</i>	<i>L. monocytogenes</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>S. enterica</i>
FGZ19I2M3	++	++	-	++	-	-	+++	-	-	-
FGZ23I1M7	++	++	-	-	-	-	+++	-	-	-
FHeNJZ3M1	++	++	-	-	-	-	+++	-	-	-
FHNFQ45M2	+	++	-	-	-	-	+++	-	-	-
FHuNCS6M8	+++	++	-	-	-	-	+++	-	-	-
FJND2M2	+++	++	-	-	-	-	+++	-	-	-
FJND16M4	+++	+	-	++	-	-	++	-	-	-
FJSYZ1M3	++	++	+	++	-	-	++	-	-	-
HeNJZ8M1	++	+	-	-	-	-	+++	-	-	-
JSSZ7M7	+++	++	-	-	-	-	+++	-	-	-
JSWX3M1	+++	++	+++	+++	-	-	+++	-	-	-
JSWX6M2	++	++	-	-	-	-	++	-	-	-
JSWX25_6	++	++	-	-	-	-	+++	-	-	-
JSWX25M6	+++	++	-	-	-	-	++	-	-	-
SDZC2M4	+++	+	++	+++	-	-	++	-	-	-
FGZ17I1M1	++	++	-	-	-	-	+++	-	-	-
FGZ19I1M3	++	+	-	-	-	-	+++	-	-	-
FGZ23I1M2	++	+	-	-	-	-	+++	-	-	-
FHeJZ44M8	+++	++	-	+++	-	-	+++	-	-	-
M203F0227	+++	++	-	+	-	-	++	-	-	-
FBJT1M6	+++	++	+++	++	-	-	+++	-	-	-
FHBSJZ9M1	+++	+	-	+	-	-	+++	-	-	-
FHBSJZ50M1	++	+	+	-	-	-	++	-	-	-
FHeBSJZ1M1	++	+	+	-	-	-	+	-	-	-
FHNFQ4M11	++	+	-	++	-	-	+++	-	-	-
FJSWX12MIM1	++	+	-	-	-	-	++	-	-	-
FJSWX14MIM1	+++	+	++	+	-	-	++	-	-	-
FLNSY3M1	+++	+	-	++	-	-	++	-	-	-
M207F01M51	++	+	-	++	-	-	++	-	-	-

^aZone size = diameter of the zone – diameter of Oxford cup in millimeters. – means no antimicrobial activity; + means 0–5 mm; ++ means 5–10 mm; +++ means 10–15 mm.

(58), and (iv) scarce class IV lanthipeptides- and sactipeptide-associated data are used for alignment. The disadvantage of bacteriocin mining *in silico* analysis is that it mainly depends on the similarity with the previously discovered BGCs, indicating the possible oversight of novel BGCs. To date, all class IV lanthipeptides are analogs of venezuelin from *Streptomyces venezuelae* (59), and only eight sactipeptides have been identified (11, 60). The number of described and characterized bacteriocins of this class is relatively small compared to that of other classes; to that effect, only two class IV lanthipeptides and six sactipeptides have been included in the BAGEL database. Zheng et al. also reported a relatively low incidence of sactipeptides (61). Therefore, we speculate that there may be novel class IV lanthipeptides and sactipeptides in *Bifidobacterium* (62). Histidine kinase and response regulator genes were identified in the sactipeptide gene cluster from *B. animalis* FJSNJLH3M11 indicating that a two-component regulatory system was subjected to sactipeptide, which had never been reported in other known sactipeptide genes.

BGCs are important genetic determinants of competitive fitness in a given niche and are frequently associated with mobile genetic elements. Competitive growth experiments in a simulated fecal environment showed that *B. longum* DJO10A outcompeted both *E. coli* and *C. difficile* more effectively than *B. longum* DJO10A-JH1 (antibiotic operon deletion strain) (50). This indicates that bacteriocin-encoding genes play an important role in intestinal niche competition. *B. infantis* has the potential to produce a tremendous diversity of bacteriocins. It mainly exists in the intestinal tract of healthy breast-fed infants and dominates the stool microbiota for a period (63). Compared to

the adult intestinal microbiome, the infant microbiome is less dense yet more diverse because of the neonatal diet of human milk. It can be assumed that dominant bacteria in the infant intestinal tract compete more effectively than the other bacteria. The homologous sequences of bacteriocins in the NCBI database can be used to understand the associations between bacteriocins and niches. Considering the comprehensiveness of bacteriocins carried by *B. infantis*, we suspect *B. infantis* to be the main source of BGCs in *Bifidobacterium*. One of the reasons that *B. infantis* rather than other *Bifidobacterium* species carrying many BGCs may be the metabolic costs. Although there are direct benefits to the host, the expression of BGCs brings more burden on the cell (64). This genetic material is transformed from a precursor peptide to a mature bacteriocin by modification, cleavage, and transport, which requires the secretion and production of numerous secondary metabolites, and the cell energy level may consequently be reduced (65). These processes require adaptive mutations or changes in nutrient supply to optimize the production of compounds (66). *B. infantis* has a tremendous number of genes involved in the metabolism of complex carbohydrates and is the only member of the *Bifidobacterium* genus that can digest all human milk oligosaccharide (HMO) structures (67). Previous studies indicate that their excellent utilization of HMOs is also the main colonization mechanism of *B. infantis* in this niche. The abundance of BGCs in the HMO-friendly *B. breve*, another dominant *Bifidobacterium* species in the infant intestinal tract, was second to that in *B. infantis*. Conversely, in some “adult-type” *Bifidobacterium* species (13), such as *B. adolescentis*, BGCs were either absent or scarce. These “adult-type” *Bifidobacterium* species lack the advantage of saccharolytic metabolism in the infant intestinal niche, while bacteriocin gene expression imposes an additional metabolic burden. It is a trade-off for other “adult-type” *Bifidobacterium* species to acquire these BGCs, as *B. infantis* and *B. breve* produce enough bacteriocins to eliminate targeted competitors. On the contrary, we hypothesized that the bacteria targeted by these bacteriocins could be potentially threatening competitors of *Bifidobacterium* in the infant intestinal niche. In the complex intestinal niche of adults, these BGCs are not sufficient to provide robust competition to the host and thus not prevalent in the “adult-type” *Bifidobacterium* species.

Several other *Bifidobacterium* strains carried bacteriocins with homology similar to bacteriocins from *Staphylococcus*, *Enterococcus*, and *Streptococcus*. The distribution of similar BGCs among different species indicates that horizontal gene transfer occurs actively among species. These three genera are also some of the first microorganisms to colonize the infant intestinal tract, accounting for a high abundance. Many studies have reported their rich production of bacteriocins (68–70). Bacteriocins are generally thought to exhibit inhibitory activities against bacterial strains that are closely related to the bacteriocin-producing bacteria strain. After searching the antimicrobial activity of those most similar bacteriocins to that from *Bifidobacterium* as reference (Table S4), many similar bacteriocins could inhibit *Enterococcus* and *Staphylococcus*. The bacteriocin bifidin I has strong anti-*Listeria* activity (51) and is homologous to propionicin SM1 which is widely expressed in the test *B. infantis*. This may partially explain the strong antibacterial activity of crude bacteriocins against *L. monocytogenes*. When comes to crude bacteriocins of test *B. infantis*, antimicrobial activities against Gram-negative bacteria are lost. In addition to the fact that other non-proteinaceous antimicrobial substances were produced by the strains, there can be two potential explanations: (i) lower pH values exert a dual effect both by promoting the production of bacteriocins (71) and by enhancing their antibacterial effects (55); additionally, (ii) compared with agar culture, the expression of bacteriocin precursor genes in broth culture was obviously repressed (29). Some research has reported the antibacterial activity of crude extracts from other *Bifidobacterium* species against *E. faecalis*, *L. monocytogenes*, and *S. aureus* (18). We speculate that *B. infantis* facilitates the predominance of *Bifidobacterium* in the infant intestinal tract by harboring BGCs that inhibit the growth of competing microorganisms. This study provides novel evidence regarding the niche competition of *Bifidobacterium*

within the infant intestinal tract that needs to be corroborated by further experimentation.

MATERIALS AND METHODS

Genome sequences and mining for BGCs

The details of the genome sequences of 759 strains belonging to *B. longum* subsp. *infantis* ($n = 36$), *B. longum* subsp. *longum* ($n = 210$), *B. breve* ($n = 146$), *B. dentium* ($n = 36$), *B. animalis* ($n = 63$), *B. angulatum* ($n = 6$), *B. bifidum* ($n = 96$), *B. adolescentis* ($n = 63$), and *B. pseudocatenulatum* ($n = 103$) are presented in Table S1. Most of these sequences were previously determined by our institution and the rest were retrieved from public data. The pairwise ANI (<https://github.com/widowquinn/pyani/>) was performed, and values were calculated and visualized to distinguish *B. longum* subsp. *infantis* and *B. longum* subsp. *longum*.

The bioinformatics tools, antiSMASH6 (20) and BAGEL4 (19), were used to mine putative BGCs, and the results (regions and areas of interest from antiSMASH6 and BAGEL4, respectively) were manually categorized. Bacteriocins were classified according to Garcia-Gutierrez et al. and Cotter et al. (3, 7). The classification of specific class I bacteriocin subgroups of gene clusters was based on the presence of highly conserved enzymes. Gene clusters with the same structural composition and order under a subgroup of BGCs were grouped into a “type.” To determine the location of precursor peptides in a particular type, all identified precursor peptides were taken into account (via comparison with the respective constructed bacteriocin databases); additionally, all proteins encoded in the gene clusters were examined using BLAST in the nr protein database to search for potential precursor peptides that may have been omitted in bacteriocin databases. A discrepancy of two or more amino acids between the identified and previously discovered bacteriocins was considered novel (72). Peptide sequences were aligned using MUSCLE (73) and visualized using Jalview (74). The similarity of pairwise sequence alignment was derived via Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). After the putative bacteriocins were described using BLAST, they were aligned with all sequences with the same description in Identical Protein Groups of NCBI, and phylogenetic analyses were constructed by IQ-TREE (75). The resulting nexus tree files were exported to iTOL for graphical adjustment (76). Most branches of the phylogenetic trees were folded for a better illustration of the evolutionary distance between the target sequence and other sequences (which may have otherwise led to more than 1,000 branches); however, this did not modify the topological structure of the phylogenetic tree.

Evaluation of the antimicrobial activity of *B. infantis* strains and their crude bacteriocins

The antimicrobial activities of 29 *B. infantis* strains from our team among 36 *B. infantis* strains were tested (Table 1). All tested strains were deposited in the Culture Collection of Food Microorganisms at Jiangnan University. Each tested strain was cultivated anaerobically for three generations in MRS liquid medium with 0.05% cysteine hydrochloride at 37°C for 24 h. After centrifugation (Eppendorf 5424R centrifuge, Hamburg, Germany) for cell removal, the supernatant was incubated with 80% saturated ammonium sulfate at 4°C overnight to obtain crude bacteriocin, which was dissolved in phosphate buffer (pH 5.5) to achieve a 20-fold concentration after desalting and then filtered through a sterilized 0.22- μm nitrocellulose membrane.

The antimicrobial activity of *B. infantis* was determined using a colony overlay assay with modifications (77). Briefly, strains were inoculated as a 3 μL spot on MRS agar plates (1.5% agar) at 37°C for 24 h under anaerobic conditions. Subsequently, cover the plate with 0.8% soft agar containing 10^7 CFU/mL indicator strain and incubated under appropriate conditions for 24 h. *Enterococcus faecalis* ATCC19433, *Staphylococcus aureus*

CGMCC1.1861, *Lactobacillus plantarum* LABCC IMAU60055, *Lactobacillus rhamnosus* ATCC7469, *B. adolescents* CGMCC 12190, *B. bifidum* CICC6071, *Listeria monocytogenes* ATCC19114, *Klebsiella pneumoniae* JNGMMA3, *Escherichia coli* CICC10413, and *Salmonella enterica* CICC21482 were included as indicator strains as they are commonly associated with the infant intestinal tract. Antimicrobial activity was evaluated based on the diameter of the inhibition zone around the spot.

The antimicrobial activity of the crude bacteriocin was determined using the Oxford cup method with modifications (78). The bottom of the plate was covered with 1.5% sterile agar, and then, 0.8% soft agar containing 10^7 CFU/mL of the indicator strain was overlaid on the plates. The Oxford cups were placed on the agar surface and lightly pressed. Next, 200 μ L of crude bacteriocin was poured into the cups. MRS liquid medium was used as a control. After 4 h of storage at 4°C, the plates were incubated under appropriate conditions at 37°C for 24 h. The antimicrobial activity of crude bacteriocins was evaluated based on the diameter of the inhibition zone from the edge of the cups. All experiments were performed in three independent replicates.

Transcription analysis

B. infantis strains were collected after 24 h of culture by centrifugation at 2,500 g for 10 min at 4°C. RNA extraction was performed using the Vazyme Bacteria RNA Extraction Kit (R403-01, Vazyme Biotech Co., Ltd., Nanjing, China). RNA integrity was analyzed by electrophoresis on a 1% agarose gel. Total RNA samples were reverse transcribed into cDNA using the Vazyme HiScript III-RT SuperMix for qPCR (R323-01). Each strain was performed in three independent biological replicates.

qPCR was used to detect the bacteriocin gene expression. The Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/Primer-Blast/>) was used to construct qPCR primers targeting bacteriocin genes in the conserved region and checked the specificity of primers (Table S2). Using the 23S rRNA gene as an internal reference gene (79), qPCR was performed in a 10- μ L mixture containing 5 μ L of 2 \times iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.5 μ L of each primer (10 nM), 1 μ L of cDNA template, and 3 μ L of deionized distilled water under the following conditions on a CFX-Connected Real-Time PCR System (Bio-Rad, Hercules, CA, USA): polymerase activation at 95°C for 30 s followed by 39 cycles, each at 95°C for 5 s and 60°C for 30 s. The amplification specificity was confirmed by melt curve analysis under the following conditions: 65°C, 5 s; 95°C, 0.5°C. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method (80). Each replicate was performed in triplicate.

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AUTHOR CONTRIBUTIONS

Di Yu, Data curation, Formal analysis, Investigation, Resources, Visualization, Writing – original draft | Zhangming Pei, Data curation, Software | Yutao Chen, Software | Hongchao Wang, Supervision, Validation | Yue Xiao, Supervision | Hao Zhang, Conceptualization, Funding acquisition, Project administration | Wei Chen, Funding acquisition, Project administration | Wenwei Lu, Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – review and editing

DATA AVAILABILITY

All sequence data of *Bifidobacterium* strains have been deposited in the NCBI GenBank database. The accession numbers for all individual genomes used in this study (including 129 genomes downloaded from NCBI) are presented in Table S1.

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Fig. S1 (AEM00979-23-S0001.pdf). Fig. S1 Transcription analysis of BGCs of the 36 *B. infantis* strains. The X-axis is the target gene whose specific name can be found in Table S2; the gray bottom indicates that the gene is not tested in the strain.

Fig. S2 (AEM00979-23-S0002.pdf). Fig. S2 Heatmap and dendrogram of ANIb values of 59 *B. longum* genomes. Sequences of 40 genomes marked as *B. longum* subsp. *infantis* (11 of which were downloaded from NCBI) and 19 genomes marked as *B. longum* subsp. *longum* (all downloaded from NCBI) were distinguished by pairwise ANIb values. ANIb, average nucleotide identity blast. The red color code refers to the strains that may be incorrectly marked based on clustering results.

Table S1 (AEM00979-23-S0003.xlsx). Table S1 Specific information of 763 *Bifidobacterium* genomes.

Table S2 (AEM00979-23-S0004.xlsx). Table S2 Primers designed and used for transcription analysis.

Table S3 (AEM00979-23-S0005.xlsx). Table S3 Specific distribution of different types of BGCs in the *Bifidobacterium*.

Table S4 (AEM00979-23-S0006.xlsx). Information summary table of bacteriocins and their most similar bacteriocins.

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