

Article

IL-4-Inducing Hypothetical Proteins of Chicken-Isolated *Limosilactobacillus reuteri*

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Abstract

Backgrounds/Objectives: Probiotics confer health-promoting and immunomodulatory effects on the host. However, a lot remains to be understood regarding their safety profile, characteristics and functional mechanisms. This study was conducted to comprehend the functional mechanisms of *Limosilactobacillus reuteri* isolated from chickens and characterize some of its uncharacterized proteins (hypothetical proteins). **Methods:** The chicken-isolated *Limosilactobacillus reuteri* genome, sequenced using Illumina MiSeq sequencing technology, was explored for proteome comparison analyses, comprehensive antibiotic resistance analysis, interleukin-4 (IL-4) expression across various conditions as well as IL-4-inducing potential. **Results:** Five antibiotic resistance-associated genes were found. Hypothetical proteins labeled QHPv.2.1, QHPv.2.12 and QHPv.2.16 induced IL-4, but QHPv.2.12 was not immunogenic. Immunogenic IL-4-inducing mutants from QHPv.2.1 (0.7013) and QHPv.2.16 (0.7040) had the best support vector machine (SVM) values, which were significantly ($p < 0.05$) higher than the rest of the group, followed by those from QHPv.2.12 (0.4587). QHPv.2.5 (0.0560) had the lowest value. QHPv.2.1 (5484.71) and QHPv.2.16 (5491.05) had significantly ($p < 0.05$) larger molecular weights (MWs), followed by QHPv.2.12 (5302.05). The lowest value was obtained for QHPv.2.7 (3386.74). **Conclusions:** This explorative study revealed the IL-4 inducing capacity of the hypothetical proteins of *L. reuteri* and their mutants, as well as identified heat and cold shock proteins associated with *L. reuteri*.

Keywords: animal nutrition; antibiotic resistance; cold shock protein; heat shock protein; gene expression; *Limosilactobacillus reuteri*; probiotics

1. Introduction

The benefits of probiotics, that is, live microorganisms which, when consumed adequately, provide health-promoting effects to both animals and humans, have inspired continuous research interest. The recent interest is focused on unraveling their functional mechanisms, as well as exploring their uncharacterized and hypothetical proteins [1].

Probiotics' functional mechanisms and their hypothetical proteins remain incompletely understood despite their documented benefits and the increasing demand for their use in animal and human nutrition. This knowledge gap has necessitated continuous research interest in completely understanding their functional mechanisms, which become clearer by the day as more research outputs document their potential functional pathways and additional benefits, including their cytokine-inducing ability, such as IL-6 induction [2] and interferon-gamma in humans and mice [3].



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The important roles that IL-4 plays in immunity cannot be overstated, including the key roles it plays in type 2 immunity, leading to toxin inactivation and resistance to helminth parasites. In a like manner, the importance of HSPs and CSPs to probiotics cannot be overstated. HSPs protect probiotics from harsh environments, such as the bile, heat and acid of the gastrointestinal tract. They protect the intestinal barrier by minimizing inflammation and enhancing as well as promoting HSPs in epithelial cells. CSPs are primarily essential in food processing involving refrigeration, enabling probiotics to withstand stress arising from food temperature, as well as enabling them to maintain functional metabolic activities.

The gut commensal microbiota, such as *Limosilactobacillus reuteri*, formerly known as *Lactobacillus reuteri*, offer structural and mechanical integrity and barrier functions to the intestinal mucosal surfaces, thus protecting the intestine [4] and maintaining a healthy immune system [5]. Of all the known commensal microbiota species used as probiotics for dietary supplements, therapeutics and cosmetic products, lactic acid bacteria (LAB) are the prominent probiotic bacteria used. Studies are ongoing not just to understand the functional mechanisms of probiotics but to also uncover new sources for developing new candidates. The starting point is usually the isolation of the potential probiotics from the feces, milk or gut of the respective animals [6]. In addition, potential candidates must not be pathogenic; they must be able to survive in the intestinal tract, possess the ability to adhere to the intestinal walls, have high vitality and be able to synthesize antimicrobial substances such as bacteriocins, as well as not be associated with antibiotic resistance. One major characteristic of LAB is their ability to produce bacteriocins.

A recent study focused on isolates (*Lactobacillus plantarum*, *Bacillus subtilis*, *Bacillus tequilensis* and *Lactobacillus fermentum*) from chickens tested for antagonistic ability, carbohydrate fermentation analysis, bile salt and low pH tolerance, hemolysis activity and intestinal adhesion, revealing that all the isolates showed great survival percentage at low pH, none exhibited a- and b-hemolytic activity and most of them showed optimal growth of up to 4% sodium chloride concentration [6]. The findings from such a study further confirmed the potential of LAB as probiotic bacteria. Another recent study reported the probiotic potential of chicken-isolated *L. saerimneri*, *L. acidophilus*, *L. ingluviei*, *L. salivarius* and *L. reuteri* [7].

Proteins that have not been experimentally assigned functions or for which translation has not been established are referred to as hypothetical proteins. It has been observed that about 2% of the genes of a genome code for protein while the remaining ones are non-coding or their functions are yet to be established [8]. Hypothetical proteins may be involved in disease development as well as help in understanding physiological and biochemical pathways such as in finding new functions, structures, pharmacological targets and markers [9,10]. Research interests are recently also focused on antibiotic-associated proteins or genes, beyond the focus on CSPs, HSPs and hypothetical proteins.

Antibiotic resistance is a global threat to human health and to the treatment of disease. In addition, antibiotic-resistant genes (ARGs) may be present in any environment including natural and engineered habitats [11], threatening public health. Probiotics are regarded as safe alternatives to antibiotics but a lot remains unexplored about their association with ARGs. Probiotics are health-promoting but their immunological mechanisms have not been fully understood, such as their relationship with IL-4. Understanding the relationships between probiotics and IL-4 is key to understanding their functional mechanisms, especially regarding hypothetical proteins. Considering the importance of LAB as probiotics and the key roles IL-4 plays in immunity, this study was conducted to understand the mechanisms underlying the functional abilities of *Limosilactobacillus reuteri* using the chicken-isolated

Limosilactobacillus reuteri genome and to characterize some of its hypothetical proteins vis-à-vis CSPs, HSPs and IL-4-inducing potential exploration.

2. Materials and Methods

2.1. Isolation and Genome Sequencing

Fecal samples from Nigeria-reared broiler chickens served as the source of bacteria isolation; Illumina MiSeq sequencing technology was adopted for the genome sequencing. The sequencing depth was 78.0× and the assembly method was SPAdes v3.14.0. For this study, the assembled complete whole genome nucleotide sequence was retrieved from NCBI (CP059275 and version.1); annotation, proteome comparison analysis and sorting of the protein families were analyzed using PATRIC. HSPs, CSPs and hypothetical proteins were selected for antibiotic resistance analysis and IL-4-inducing potential gene expression. Table 1 shows the selected studied proteins.

Table 1. Investigated selected proteins from *L. reuteri* genome.

Protein	Designated As
* 1674 hp	** 2.1
* 1606 hp	** 2.2
* 1944 hp	** 2.3
* 2105 hp	** 2.4
* 2195 hp	** 2.5
* 218 hp	** 2.6
* 2256 hp	** 2.7
* 2313 hp	** 2.8
* 2318 hp	** 2.9
* 284 hp	** 2.11
* 27 hp	** 2.12
* 357 hp	** 2.13
* 34 hp	** 2.14
* 37 hp	** 2.15
* 1674 hp	** 2.16
* 1606 hp	** 2.17
* 365 CSP ⁺	C1
* 1253 HSP ¹⁰	H10
* 1252 HSP ⁶⁰	H60
* 1668 HSP ^g	Hg
* 1900 HSP ^h	HX
* 703 CSP ⁺	C2

* fig | 1598.3022.peg.; hp = hypothetical protein; HSP^g = heat shock protein GrpE; HSP^h = heat shock protein HtpX; HSP¹⁰ = 10 kDa family chaperone GroES; HSP⁶⁰ = 60 kDa family chaperone GroEL; CSP⁺ = cold shock protein of CSP family; ** = QHPv.; H10 = HSP10 kda; H60 = HSP60 kda; Hg = HSPGrpE; HX = HSPHtpX; C1 = CSP1; C2 = CSP2.

2.2. BLAST (2.17.0) and Multiple Sequence Alignment

The HSPs and CSPs found in the *L. reuteri* genome were analyzed for an amino acid sequence similarity check using NCBI protein–protein blast and Blast Tree View was used to generate the trees for selected organisms: *Homo sapiens* (taxid:9606), *Mus* (taxid:10088), chicken (taxid:9031), Wistar rats (taxid:10116) and *Rattus norvegicus* (taxid:10116). Multiple sequence alignment of the heat and cold shock proteins was carried out with Clustal Omega (1.2.4), <https://www.ebi.ac.uk/jdispatcher/msa/clustalo> (accessed 17 July 2024), based on seeded guide trees and a hidden Markov model profile–profile approach for generating the presented alignments.

2.3. Antibiotic Resistance Analysis

A comprehensive antibiotic resistance analysis of the chicken-isolated *L. reuteri* genome was conducted using the Comprehensive Antibiotic Resistance Database (<https://card.mcmaster.ca/analyze/rgi>, accessed 17 July 2024). The RGI criteria used were perfect, strict and complete genes only. The detection criterion used was protein homolog model.

2.4. IL-4-Inducing Proteins and Mutants

The IL-4-inducing capacity of the selected hypothetical proteins *Limosilactobacillus reuteri* was investigated using IL4pred (<https://webs.iitd.edu.in/raghava/il4pred/design.php>, accessed 17 July 2024). Thereafter, we designed 400 mutants from the aforementioned proteins. The mutants were designed with a single amino acid amendment for each mutant. The IL-4-inducing potential of the mutants were assessed as previously described. Support vector machine (SVM) scores were used for grouping the mutants, with the top fifty being selected and evaluated for immunogenicity using default thresholds. After this, the top fifteen (out of fifty) were assessed and immunogenic mutants with IL-4-inducing potential were statistically analyzed.

2.5. Expression of Genes

GENEVESTIGATOR was used for IL-4 gene expression and co-expression analyses (*Homo sapiens* and *Mus musculus*) considering different conditions, according to HS_AFFY_U133PLUS_2-0 (human) and MM_AFFY_430_2-1 (mouse) platforms. The expression of HSPs and CSPs across various experimental sets in *Rattus norvegicus* were also determined in GENEVESTIGATOR using the platform RN_AFFY_230_2-1. While RNA-seq may have offered a different result, Affymetrix microarray datasets were used for sample availability, cross-study comparability and a focus on differential expression.

2.6. Analysis of Statistics

Organization of the top fifteen mutants with IL-4-inducing potential was done according to completely randomized design (CRD). The data were analyzed according to one-way analysis of variance, using SAS (SAS 9.4) GLM. Duncan's multiple range test was used for separating significant means. Statistical significance was set at $p < 0.05$.

3. Results

The chicken-isolated *L. reuteri* genome had one contig, with a length of 2,085,667 bp and an average G + C content of 39.43%. It had 2471 protein-coding sequences (CDSs), 78 transfer RNA (tRNA) genes, and 10 ribosomal RNA (rRNA) genes. A circular view of the genome, a circular graphical display of the distribution of the genome annotations, the subsystems unique to the genome, the proteome comparison analysis of similar genomes and the phylogenetic tree are shown in Figure 1a–e.

In total, 1485 proteins with functional assignments and 986 hypothetical proteins were reported for the genome. The proteins with functional assignments included 586 proteins with Enzyme Commission (EC) numbers, 506 with Gene Ontology (GO) assignments and 422 proteins mapped to KEGG pathways. The circular graphical display of the distribution of the genome annotations included from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content and GC skew. The subsystems the genes belong to are represented with the colors of the CDS on the reverse and forward strands as indicated in the subsystem chart.

The HSP and CSP amino acid sequence similarity check of the genome was carried out in NCBI. The Blast Tree View was used to generate the trees for selected organisms,

and they are presented in Figure 2a–d. There were no significant alignments for HtpX (fig | 1598.3022.peg.1900) and CSP of the CSP family (fig | 1598.3022.peg.703). The results of the multiple sequence alignment (MSA) carried out among HSPs and CSPs are presented in Figure 2e,f.

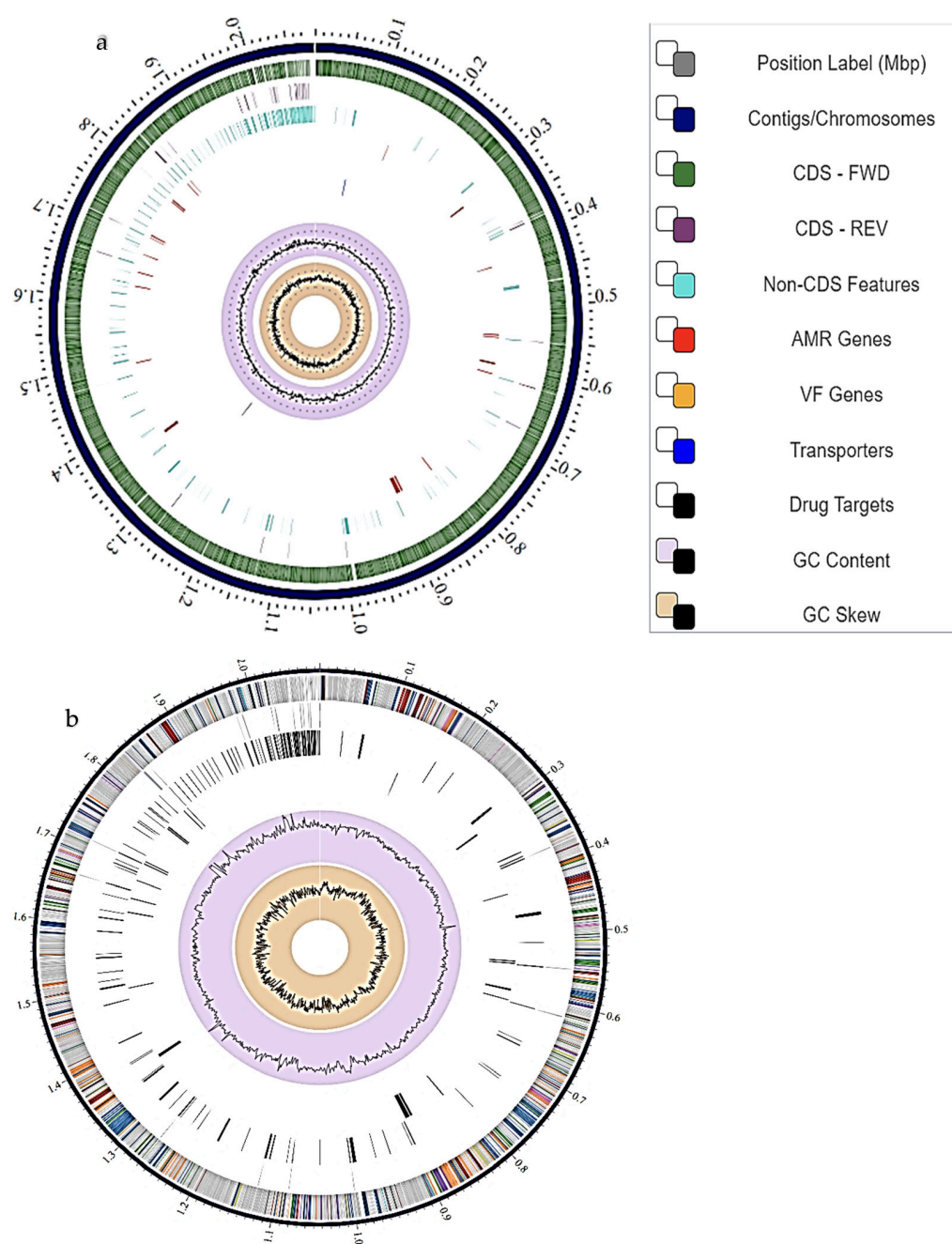


Figure 1. Cont.

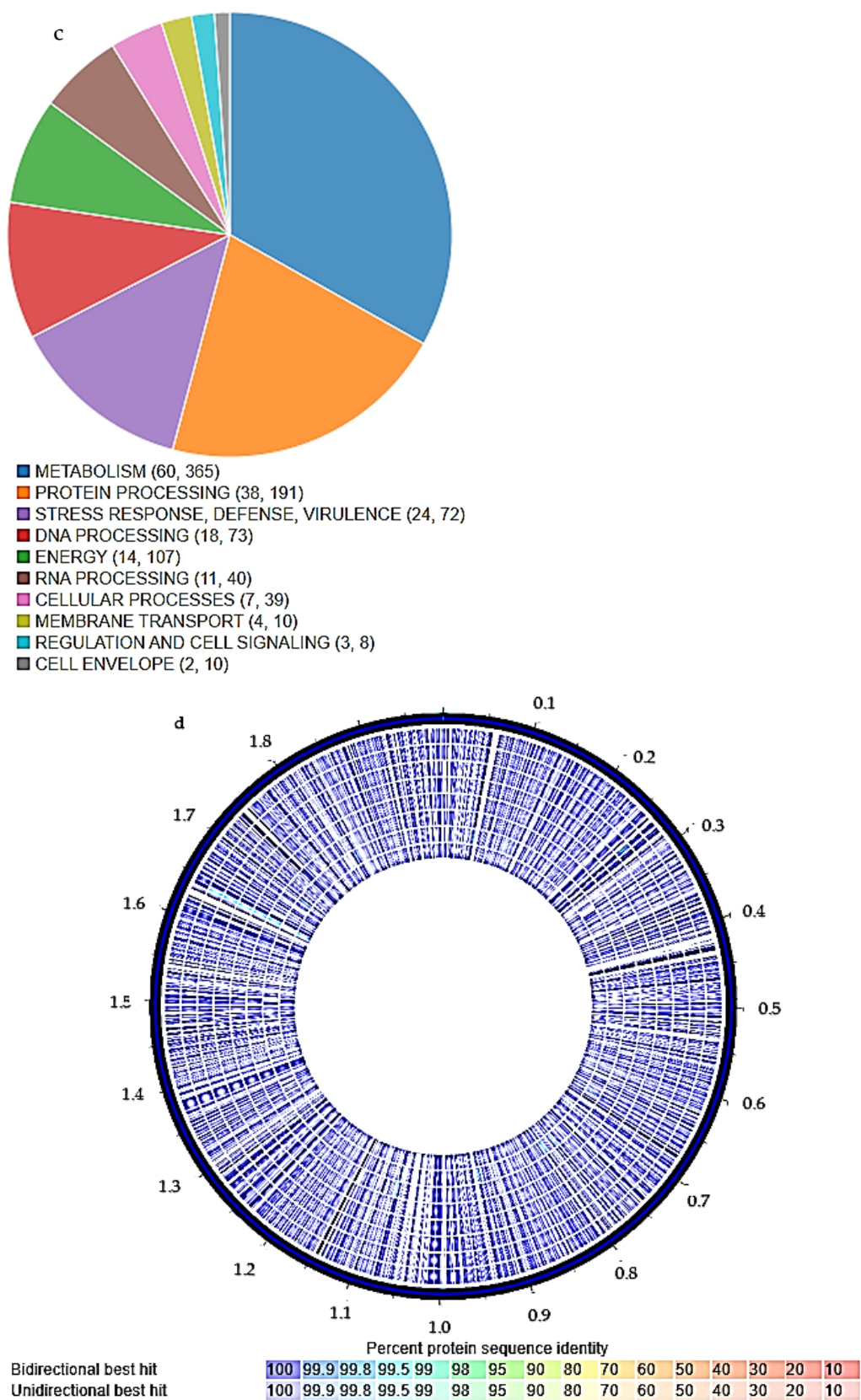


Figure 1.
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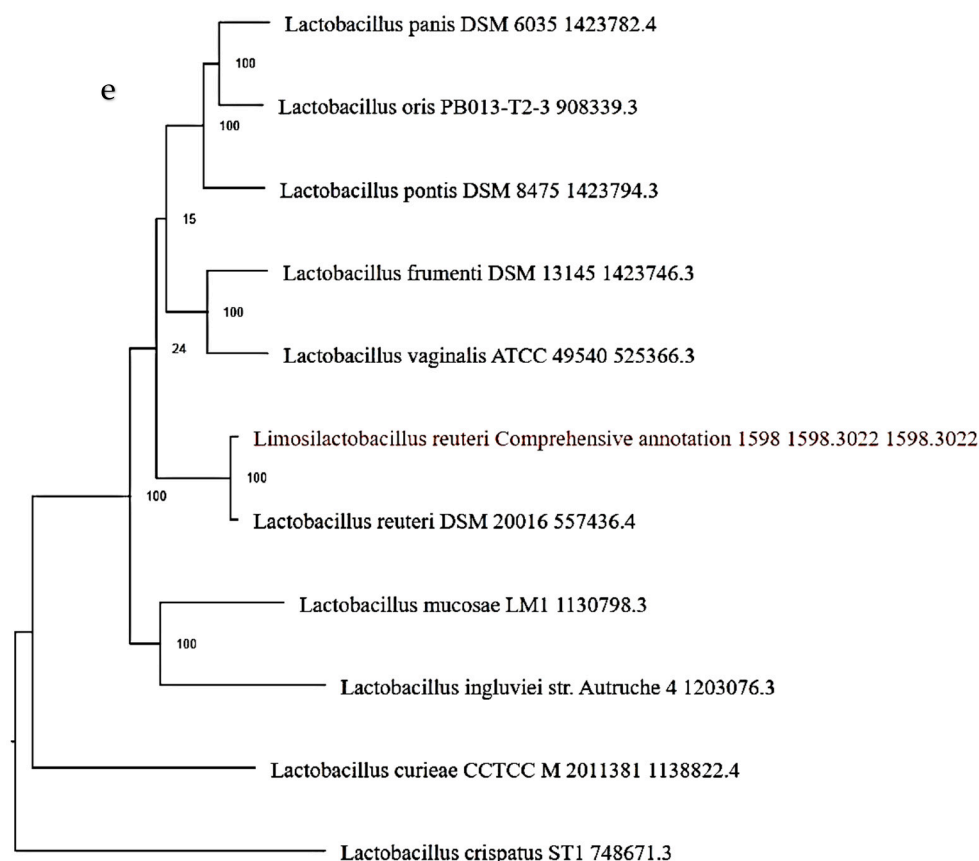


Figure 1. Annotation results of the chicken-isolated *L. reuteri* genome; (a) circular view of the genome, (b) circular graphical display of the distribution of the genome annotations of the chicken-isolated *L. reuteri* genome, (c) a chart showing the subsystems unique to the chicken-isolated *L. reuteri* genome, (d) circular view of the proteome comparison of similar genomes to chicken-isolated *L. reuteri* genome—1. *Lactobacillus acidophilus* 1579cga (1579.814), 2. *Lactobacillus acidophilus* La-14 1314884 (1,314,884.31), 3. *Lactobacillus acidophilus* strain ATCC 53544 (1579.81), 4. *Lactobacillus acidophilus* strain FSI4 (1579.28), 5. *Lactobacillus acidophilus* strain LA-G80-111 (1579.364), 6. *Lactobacillus acidophilus* NCFM (272,621.13), 7. *Lactobacillus acidophilus* strain DSM 20079 (1579.223), 8. *Lactobacillus acidophilus* La-14 (1,314,884.3)—and (e) phylogram of the chicken-isolated *L. reuteri* genome.

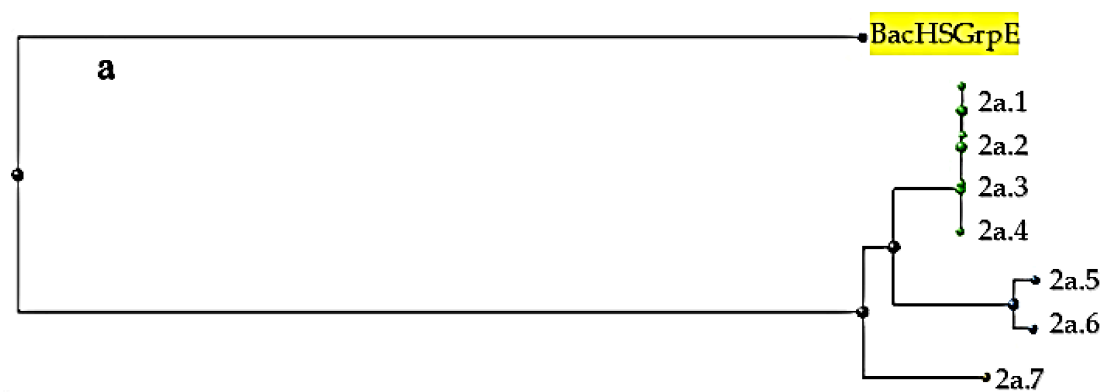


Figure 2. Cont.

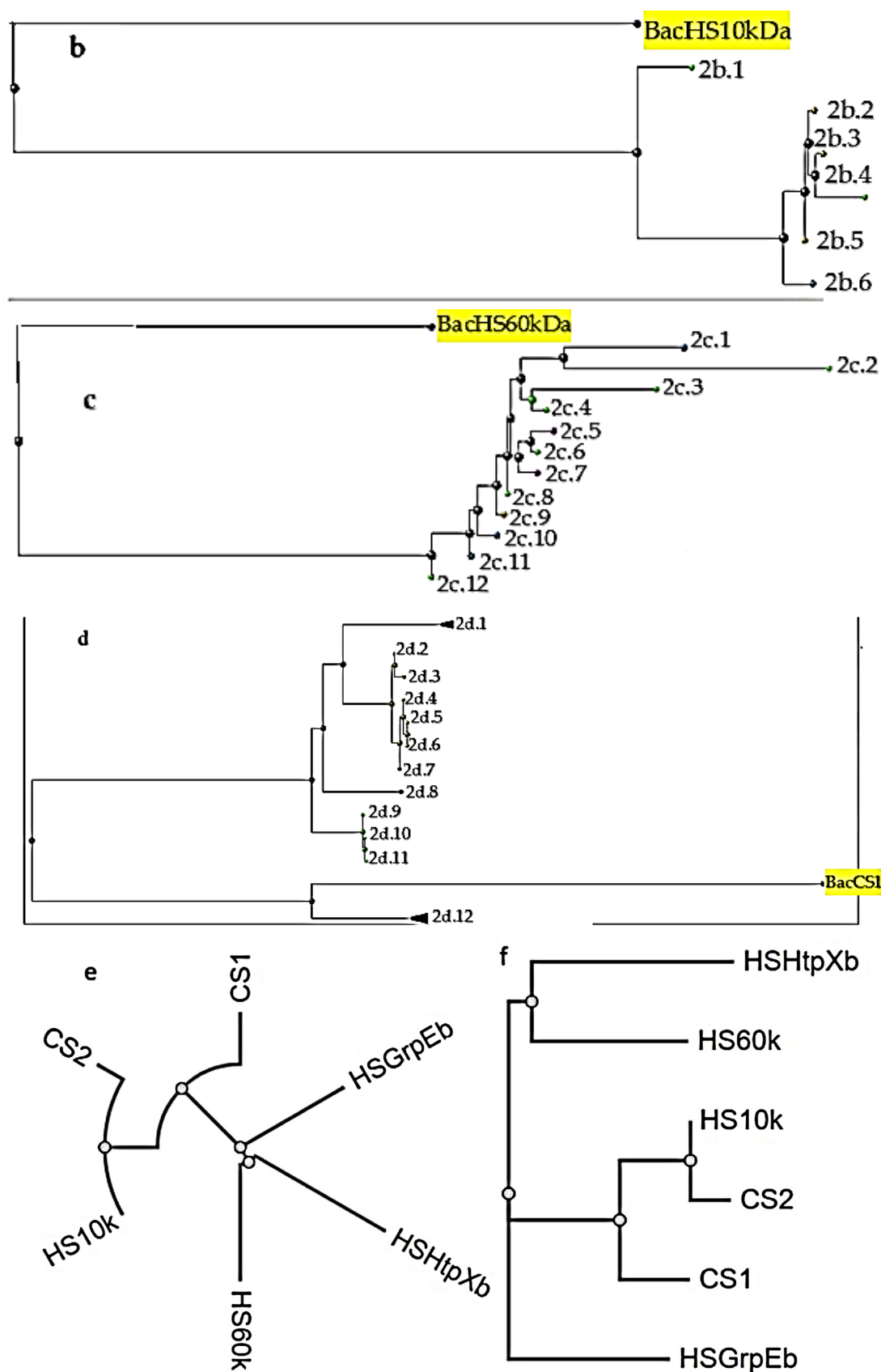


Figure 2. Blast Tree View result for protein–protein blast for chicken-isolated *L. reuteri* genome: (a) heat shock protein GrpE (fig|1598.3022.peg.1668), (b) HSP 10 kDa family chaperone GroES (fig|1598.3022.peg.1253), (c) HSP 60 kDa family chaperone GroEL (fig|1598.3022.peg.1252), (d) CSP of CSP family (fig|1598.3022.peg.365), (e) radial view of multiple sequence alignment for the query

heat and cold proteins, (f) a tree connecting the query heat and cold proteins arising from multiple sequence alignment. 2a.1 = predicted grpE protein homolog 1, mitochondrial isoform X2 (*Mandri leucophaecus*); 2a.2 = unnamed protein product (*Homo sapiens*); 2a.3 = grpE protein homolog 1, mitochondrial precursor (*Pongo abelii*); 2a.4 = predicted grpE protein homolog 1, mitochondrial isoform X1 (*Cercopithecus atys*); 2a.5 = grpE protein homolog 1, mitochondrial precursor (*Mus musculus*); 2a.6 = predicted grpE protein homolog 1, mitochondrial isoform X1 (*Rattus norvegicus*); 2a.7 = grpE protein homolog 1, mitochondrial (*Gallus gallus*); 2b.1 = CPN 10-like protein (*Mus musculus*); 2b.2 = heat shock 10 kDa protein (chaperonin 10), isoform CRA_g (*Homo sapiens*); 2b.3 = Chain 1, kDa heat shock protein, mitochondrial (*Homo sapiens*); 2b.4 = 10 kDa heat shock protein, mitochondrial (*Mus musculus*); 2b.5 = heat shock 10 kDa protein (chaperonin 10), isoform CRA_f (*Homo sapiens*); 2b.6 = heat shock family E (HSP 10) member 1 (*Myotis myotis*); 2c.1 = low quality protein: 60 kDa heat shock protein mitochondrial-like (*Mus pahari*); 2c.2 = hCG33148 (*Homo sapiens*); 2c.3 = 60 kDa chaperonin (*Homo sapiens*); 2c.4 = 60 kDa chaperonin (*Homo sapiens*); 2c.5 = heat shock protein family D (HSP 60) member 1 (*Nyctis myotis*); 2c.6 = unnamed protein product (*Homo sapiens*); 2c.7 = heat shock protein family D (HSP 60) member 1 (*Phyllotomus discolor*); 2c.8 = 60 kDa heat shock protein, mitochondrial (*Homo sapiens*); 2c.9 = 60 kDa heat shock protein, mitochondrial (*Camelus dromeda*); 2c.10 = mCG116284 (*Mus musculus*); 2c.11 = 60 kDa heat shock protein, mitochondrial (*Mus pahari*); 2c.12 = unknown (*Homo sapiens*); 2d.1 = multiple organisms, 6 leaves; 2d.2 = rCG58531, isoform CRA_b (*Rattus norvegicus*); 2d.3 = protein lin-28 homology B isoform X2 (*Grammomys surdaster*); 2d.4 = protein lin-28 homolog B (*Mus musculus*); 2d.5 = lin-28 homolog B (*C. elegans*), isoform CRA_a (*Mus musculus*); 2d.6 = unnamed protein product (*Mus musculus*); 2d.7 = protein lin-28 homolog B isoform X1 (*Mus musculus*); 2d.8 = protein lin-28 homolog B isoform (*Gallus gallus*); 2d.9 = protein lin-28 homolog B (*Homo sapiens*); 2d.10 = lin-28 homolog B (*Homo sapiens*); 2d.11 = chain A, protein lin-28_homolog B (*Homo sapiens*); 2d.12 = multiple organisms, 33 leaves.

Figure 3 shows the result of the comprehensive antibiotic resistance analysis of the chicken-isolated *L. reuteri* genome. Five antibiotic resistance-associated gene families were found only under strict criterion: glycopeptide resistance gene cluster, vanT; chloramphenicol acetyltransferase (CAT); lincosamide nucleotidyltransferase (LNU); tetracycline-resistant ribosomal protection protein and Erm 23S ribosomal RNA methyltransferase. The glycopeptide resistance gene cluster, vanT (vanT), belonged to the glycopeptide antibiotic drug class, having a 33.87% identity-matching region. Its resistance mechanism was antibiotic target alteration with a 52.67% length of reference sequence. CAT belonged to the phenicol antibiotic drug class, having a 95.35% identity-matching region; its resistance mechanism is antibiotic inactivation, with a 100% length of reference sequence. Erm 23S ribosomal RNA methyltransferase (ErmB) belonged to macrolide, lincosamide, streptogramin, streptogramin A and streptogramin B antibiotics, with a 98.39% identity-matching region; its resistance mechanism was antibiotic target alteration, with a 100% length of reference sequence. Tetracycline-resistant ribosomal protection protein (tet(W)) belonged to the tetracycline antibiotic drug class; its resistance mechanism was antibiotic target protection, having a 96.24% identity-matching region with a 100% length of the reference sequence. Lincosamide nucleotidyltransferase (LNU) belonged to the lincosamide antibiotic drug class; its resistance mechanism was antibiotic inactivation, having a 97.52% identity-matching region with a 100% length of reference sequence.

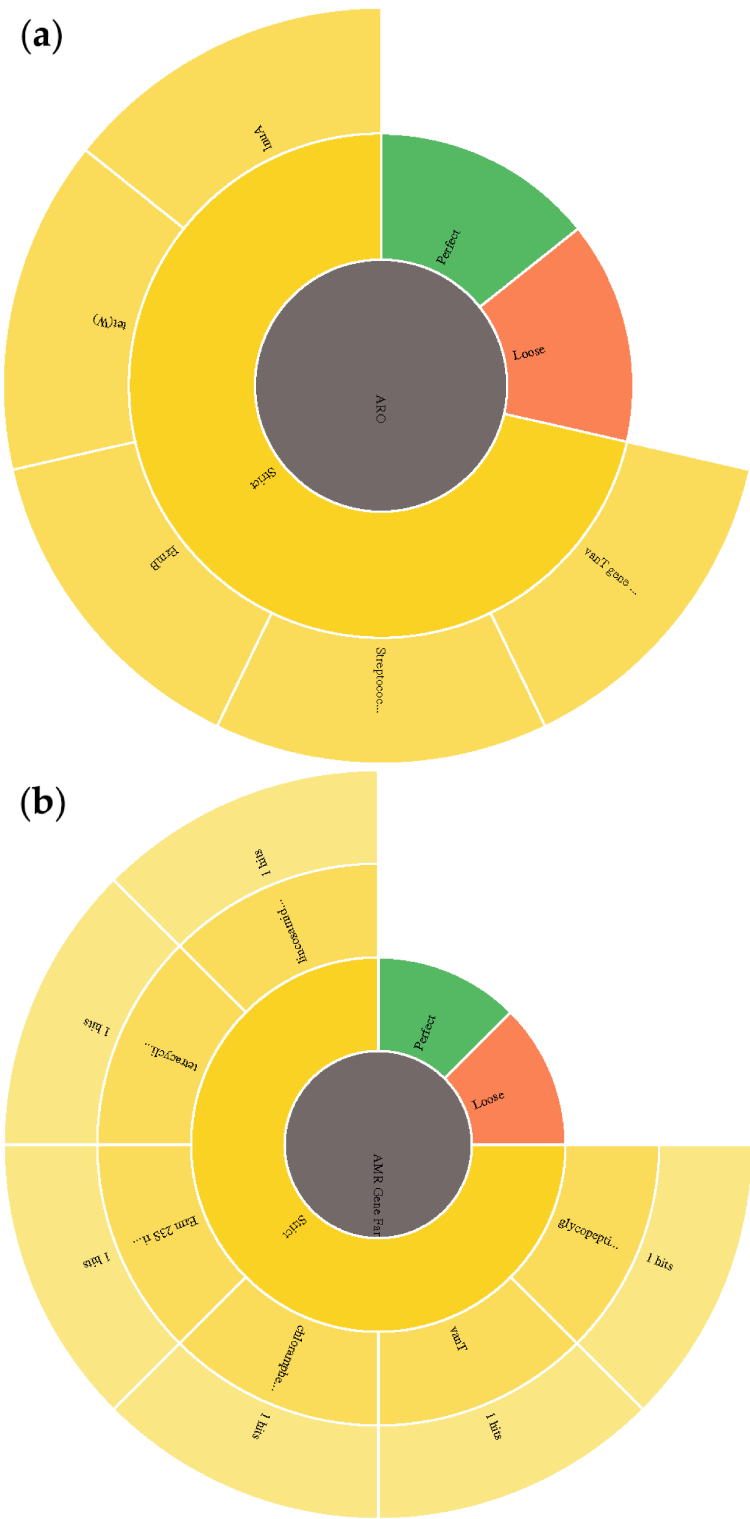


Figure 3.
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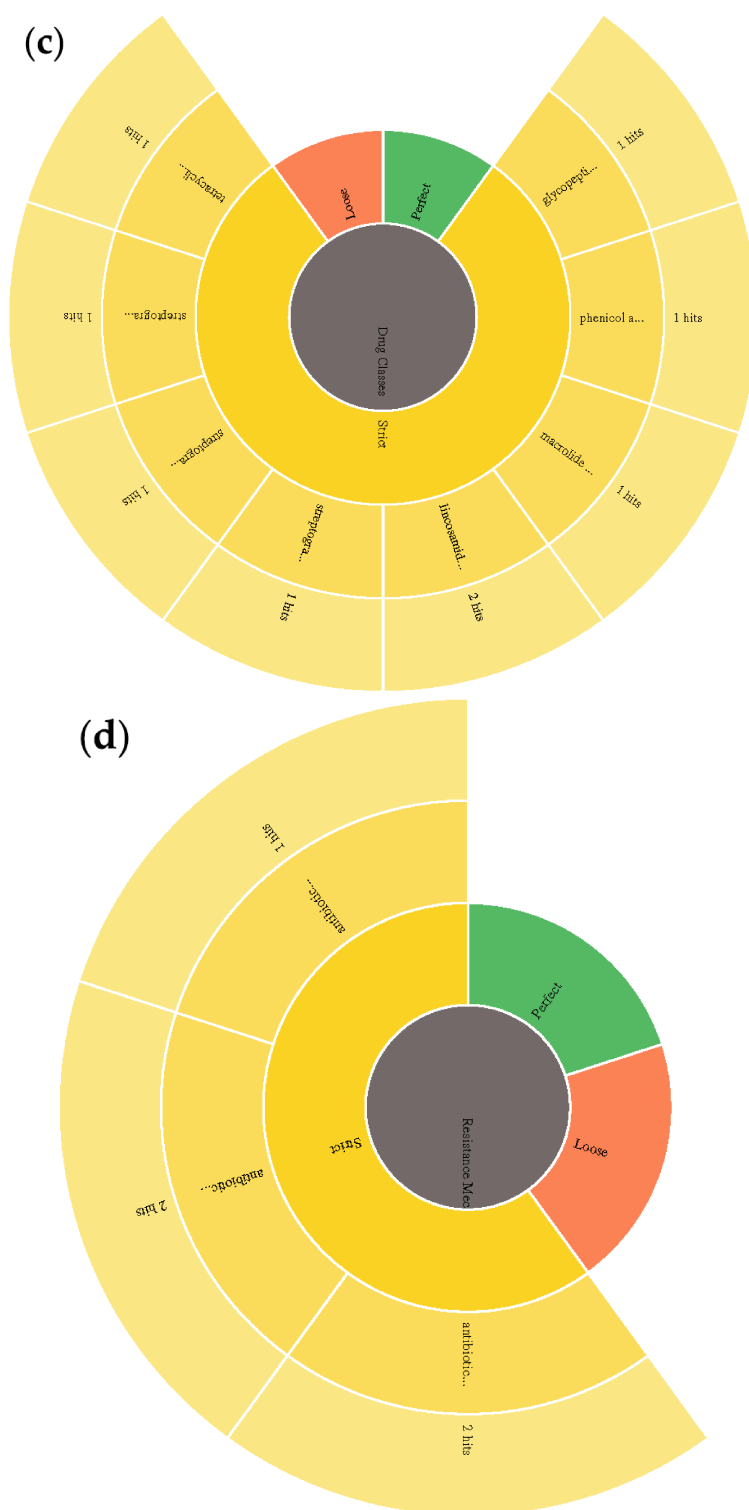


Figure 3. Comprehensive antibiotic resistance analysis of chicken-isolated *L. reuteri* analyzed with the Comprehensive Antibiotic Resistance Database, showing the (a) AMR gene, (b) AMR gene family, (c) drug class and (d) resistance mechanism. AMR Gene Fam... = AMR Gene Family; 3a: van T gene... = van T; streptococ... = streptococcus; 3b: Tetracyclin... = tetracycline-resistant ribosomal 1 protection protein; Lincosamide... = lincosamide nucleotidyltransferase (LNU); Chloramphe... = chloramphenicol; Glycopepti... glycopeptide resistance gene cluster; Erm 23s ri... = Erm 23s ribosomal RNA methyltransferase; 3c: glycopepti... = glycopeptide antibiotics; phenicol a... = phenicol antibiotic; macrolide... = macrolide antibiotic; lincosamid... = lincosamide antibiotic; streptogra... = streptogramin antibiotic; tetracycli... = tetracycline antibiotic; 3d: Resistance mec... = resistance mechanism; antibiotic... = antibiotic inactivation.

The IL-4-inducing hypothetical proteins (QHPv.2.1, QHPv.2.12 and QHPv.2.16) are shown in Figure 4, according to immunogenicity and SVM scores. Out of the three IL-4-inducing proteins, QHPv.2.12 was not immunogenic. The SVM values for QHPv.2.1 and QHPv.2.16 were 32% higher than QHPv.2.12.

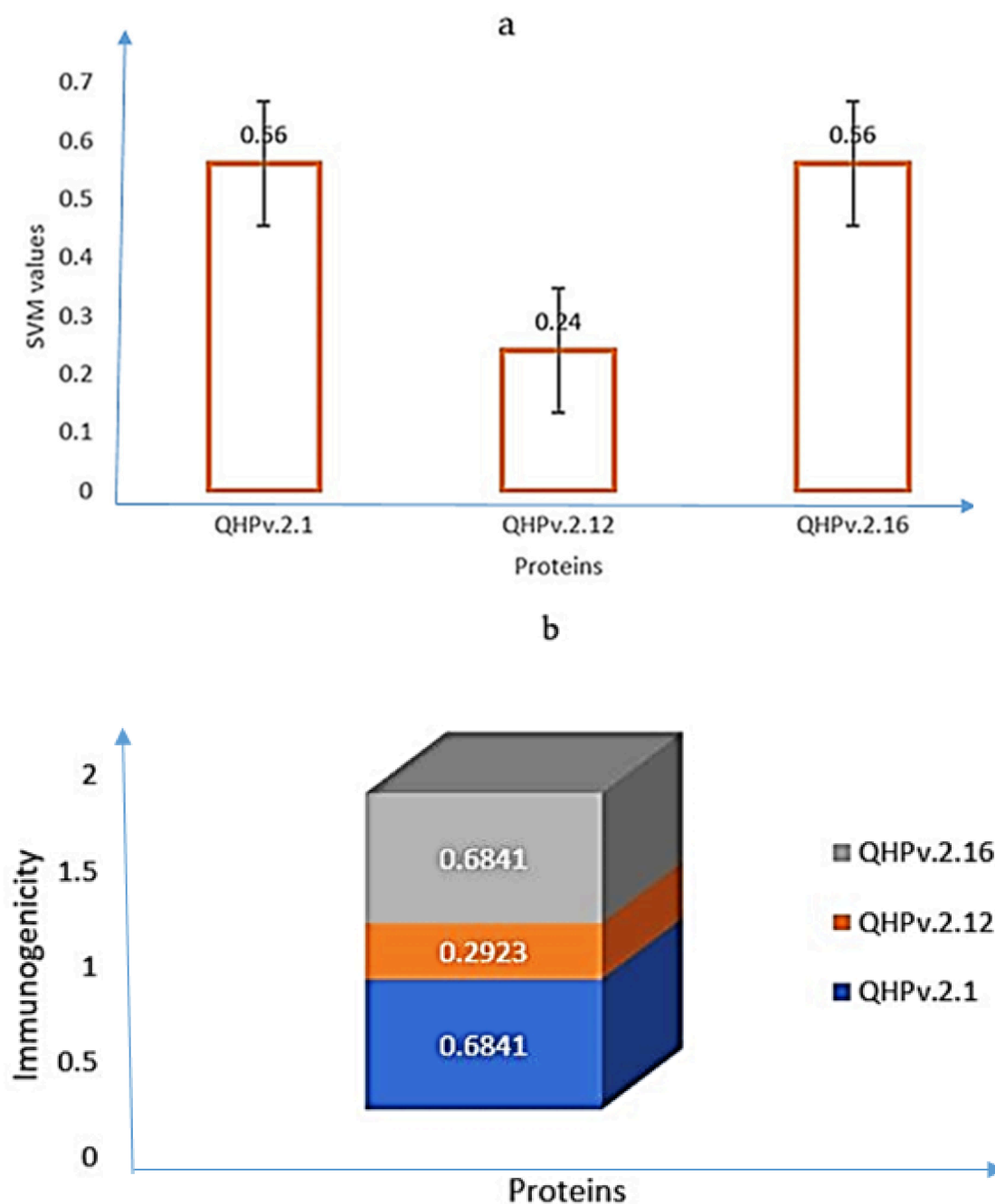


Figure 4. Hypothetical proteins with IL-4-inducing potential of the explored bacterial genome using (a) support vector machine score and (b) antigenicity value. The SVM values for QHPv.2.1 and QHPv.2.16 were 32% higher than the value obtained for QHPv.2.12 but QHPv.2.12 was not immunogenic.

The top fifteen IL-4-inducing mutants from each group, based on their immunogenicity and SVM scores were statistically analyzed. Table 2 shows the statistics of chicken-isolated *Limosilactobacillus reuteri* immunogenic hypothetical proteins and mutants that induced IL-4.

Table 2. Statistical analysis result of immunogenic mutants with IL-4 inducing potential of the explored genome.

MS	Sv (IL-4)	Molecular Weight	pI	IS
QHPv.2.1	0.7013 ^a	5484.71 ^a	7.917 ^a	0.7461 ^c
QHPv.2.5	0.0560 ^e	4987.75 ^c	6.942 ^b	1.1673 ^b
QHPv.2.7	0.2680 ^c	3386.74 ^e	4.445 ^c	1.3380 ^a
QHPv.2.8	0.2328 ^d	4506.88 ^d	8.480 ^a	1.2905 ^a
QHPv.2.12	0.4587 ^b	5302.05 ^b	4.212 ^c	0.4377 ^d
QHPv.2.16	0.7040 ^a	5491.05 ^a	7.828 ^a	0.7407 ^c
SEM	0.0262	79.47	0.21	0.0136

MS = mutant source; Sv = SVM value; pI = isoelectric point; IS = immunogenicity score; SEM = standard error (pooled) of the mean. Means that have different superscripts in the same columns are implied to be significantly ($p < 0.05$) different.

The immunogenic IL-4-inducing mutants from QHPv.2.1 (0.7013) and QHPv.2.16 (0.7040) had the best SVM values, which were significantly ($p < 0.05$) higher than the rest of the group, followed by those from QHPv.2.12 (0.4587). QHPv.2.5 (0.0560) had the lowest value. QHPv.2.1 (5484.71) and QHPv.2.16 (5491.05) had the largest molecular weights followed by QHPv.2.12 (5302.05). The lowest value was obtained by QHPv.2.7 (3386.74). QHPv.2.1 (7.917) and QHPv.2.16 (7.828) obtained higher theoretical isoelectric points compared with other mutants. QHPv.2.7 (4.445) and QHPv.2.12 (4.212) had the lowest values. According to immunogenicity value, QHPv.2.7 (1.3380) and QHPv.2.8 (1.2905) had the highest values, which were significantly ($p < 0.05$) higher than the values obtained by other mutants, followed by QHPv.2.5 (1.1673) and then QHPv.2.12 (0.4377).

Table 3 shows the corresponding position, theoretical isoelectric points and molecular weights of the selected top three immunogenic mutants with IL-4-inducing capacity from the explored bacterial genome.

Table 3. Selected immunogenic mutants with IL-4-inducing potential.

PS	Pseq	Mutant Position	pI	MW
QHPv.2.1	MNKESTVNSNSQSKIETIHASIDPKATEEEREIEIKRIENLKKQKQK	37	8.59	5482.89
	MNKESTVNSNSQSKIETIHASIDPKATEEEREIEIKWIENLKKQKQK	41	8.56	5527.93
	MNKESTVNSNSQSKIETIHASIDPKATEEEGEEIEIKWIENLKKQKQK	31	5.78	5413.79
	MNKESTVNSNSQSKIETIHISIDPKATEEEREIEIKWIENLKKQKQK	20	7.12	5555.01
QHPv.2.7	MIKTDKDTKTLEAQAVDLELRLNIDLEH	18	4.65	3367.33
	MIKTDKDTKTLEAQAVDFELRLNIDLEH	18	4.65	3401.34
	MIKTDKDTKTLEAQAVDEELRLNIDLEF	29	4.29	3393.31
	MIKTDKDTKTLEAQAVDEELRLNIDLEH	6	4.29	3358.24
QHPv.2.8	MPNWFAIFGMVFGFVILPLILFWLTRVERAENDERVK	27	6.6	4456.91
	MPNWFAIFGMVFGFVILPLILFWLTRRVRAENDERVK	28	10.68	4483.98
	MPNWFAIFGMVFGFVILPLILFWLTRIERAENDERVK	27	6.6	4470.94
	MPNWFAIFGMVFGFVILPLILFWGTRRERAENDERVK	24	9.09	4457.85
QHPv.2.16	MNKESTVNSNSQSKIETIHASIDPKATEEEREIEIKWIENLKKQKQK	41	8.56	5527.93
	MNKESTVNSNSQSKIETIHASIDPKATEEEGEEIEIKWIENLKKQKQK	31	5.78	5413.79
	MNKESTVNSNSQSKIETIHISIDPKATEEEREIEIKWIENLKKQKQK	20	7.12	5555.01
	MNKESTVNSNSQSKIETIHASIDPKATEEEREIEIKWIENLKKQKQK	32	8.59	5496.97

PS = protein source; Pseq = protein sequence; pI = isoelectric point; MW = molecular weight.

We used cross-species IL-4 gene expression in *Homo sapiens* (human) and *Mus musculus* (mouse) using GENEVESTIGATOR because of the limitation of public chicken transcriptomic data for IL-4 under our study condition, as well as to enable a broader overview into the mammalian system, considering the broader application of probiotics in animal nutrition, food science and technology, health care and therapeutic industries. IL-4 was

upregulated in the blood, peripheral, bone marrow, ovary, tumor, lung, temporal lobe and in the following cell types: CD4 helper T-cell, oogonium, CD8, cytotoxic T-cell, CD8 T-cell, myeloid cell, CD4 memory T-cell, CD8 T-cell, CD8 memory T-cell and CD4 regulatory T-cell as shown in Figure S1a,c. The IL-4 expression level was low or average in various cancer conditions (Figure S1b). Figure S1d,e show the top 50 positive genes co-expressed with IL-4 under different cells, anatomy, cancer categories and experimental sets. For instance, untreated memory CD4 T-cell samples compared with memory T-cell activation resulted in the upregulation of IL-4 (Figure S1f). In terms of the cell line, IL-4 was upregulated in 32D-BCR/ABL-ESR-Cebpa (mouse), but the expression level (Log2 ratio) was low or average across other cell lines (Figure S1g). The positive co-expression of genes with IL-4 according to cancer categories in mice is presented in Figure S1h. The 4-hydroxytamoxifen treatment for 6 h, 9 h and 12 h against control treatments resulted in the upregulated expression of IL-4 in mice (Figure S1i). Figure S1j shows the hierarchical clustering of IL-4 and IL-4ra in mice based on anatomical parts, cell types and cancer conditions. Figure S1k presents the expression level of HSPs and CSPs in *Rattus norvegicus* across various perturbations. For instance, Carhsp1 was upregulated in soman study 1 (48 h; untreated hippocampus tissue), sarin-induced seizures (24 h; 2-PAM, atropine sulfate, diazepam; L5; ipsilateral), and neuropathic pain study 1 (72 h; untreated hippocampus tissue).

4. Discussion

In this explorative study, we made attempts to understand the properties of the *L. reuteri* genome by exploring its HSPs, CSPs, hypothetical proteins and their mutants in relation to their IL-4-inducing capacity, considering the key roles IL-4 plays in immunity as well as its conserved nature. Probiotics and fermented foods have been extensively studied. The benefits of microbial and fermented food or feed in human and animal nutrition keep unfolding, and the market for microbial probiotics keeps growing. The benefits of probiotics include improved growth performance, feed conversion efficiency, immune responses, combating enteric pathogens and modulation of the intestinal microbiota [12,13]. Nonetheless, research efforts are ongoing to understand how probiotics perform its immune-boosting functions among other key roles, necessitating studies like this.

This study and its findings are of interest because it has been documented that a high population of probiotic bacteria in the intestinal tract can form colonies that protect the digestive tract by sticking to the epithelial tissue on the enterocyte wall and reducing the formation of pathogenic bacteria colonies [14,15]. Unraveling the mystery of how these functions are performed is key and requires concerted efforts to enable our understanding of probiotics in their entirety and their associated threats, if any.

Based on the documented benefits of probiotics and recent findings unfolding their ability to induce cytokines [16–18], which may improve immunity in human and animals as corroborated by the findings in this study, researchers continue to seek cost-effective and safe probiotic candidates for use as feed additives, food supplements and cosmetics and for therapeutic purposes. Kassa et al. [6] recently presented 12 potential probiotic bacteria from selected indigenous Ethiopian chicken gastrointestinal tracts, indicating a growing interest in microbial probiotics for human and animal consumption. The best probiotic candidates reported by the authors were the isolates GCH212 and GCM412, belonging to *L. plantarum* and *B. subtilis*. Probiotic bacteria from the native host are preferred to those isolated from other sources [19–21].

Efforts to identify the best probiotic candidates have suggested isolating probiotic strains from species they are fed to, such as chickens and pigs. A recent study adjudged strains of *Lactobacillus* obtained from contents of chicken guts to have probiotic potential and be safe [7]. The bile salt tolerance analysis of *Lactobacillus* strains isolated from the

digestive tract of chickens in different bile salts revealed that *L. reuteri* had the best survival rates for both 0.30% and 1.00% bile salt, indicating its probiotic potential. However, a lot remains unclear about the functional mechanisms of these beneficial organisms.

In the current study, HSPs and CSPs were identified, five genes were associated with antibiotic resistance, and three of the hypothetical proteins including some of their mutants induced IL-4. To the best of our knowledge, this is the first study showing this kind of result thus far, thereby shedding light on the functional mechanisms of this beneficial microorganism and laying a foundation for further in vivo studies, which may benefit poultry and swine industries, food and dietary supplements as well as therapeutic industries.

Exploring HSPs and CSPs are of specific interest because extreme temperature is usually adopted to preserve food by inhibiting microbial growth, chemical reactions, autolytic enzyme activity and invasion. Such a method may also have negative impacts on beneficial microorganisms such as probiotics. In the present study, CSPs and HSPs present in *L. reuteri* strongly suggest that *L. reuteri* may be able to withstand such extreme temperatures, thereby preserving their beneficial effects for the host, although this is an explorative study that has not validated that claim with wet-lab experimental findings. The suggested benefit is based on the general knowledge that bacteria respond to the cold by preventing RNA from forming a stable secondary structure and promoting transcription and translation [22]. Two genes associated with the cold shock protein (CSP) of the CSP family were identified in the genome of chicken-isolated *L. reuteri* in the present study, which may need to be further explored for their specific functions.

Based on the present knowledge, CSPs play significant roles in bacterial cold adaptation and are induced under conditions of temperature drift. They are highly conserved small proteins, composed of nucleic acid-binding cold shock domains, which can bind to either a single-stranded DNA or RNA or as a chaperone [23]. The presence of one or more nucleic acid-binding cold shock domains endows them with pleiotropic functions, such as the regulation of splicing, transcription and translation [24,25]. They were previously reported in some foodborne pathogenic bacteria and food spoilage bacteria [26–28] but studies reporting their presence in probiotic bacteria are rare, as reported in this study.

CSPs play crucial roles in bacterial cellular functions, including differentiation and normal growth, virulence, starvation and other physiological and biochemical processes [29], making them ideal for growth, recovery and other bacterial cell functions at low temperatures, although CSPs in probiotic bacteria are rarely studied. CSPs are a ‘jack-of-all-trades’. They are conserved in both structure and function and can be found in almost all organisms from bacteria to humans (except yeast). Also, they have been detected in almost every cellular compartment. They can regulate their expression and several disease-associated genes and orchestrate multiple cellular processes [30]. As the authors [30] admitted, there is a lot to be conducted in characterizing and understanding the full cell-specific functions and mechanisms of CSPs.

Both CSPs and HSPs were identified in the studied genome in this study. Like CSPs, HSPs are ‘stress proteins’ that are highly conserved. They are a special group among chaperones and play important roles in protein folding and structuring as well as in maintaining their functional conformations, assembling multi-protein complexes and preventing non-native protein aggregation [31]. They improve membrane stability and assist during the reactive oxygen species detoxifying process by positively regulating the antioxidant enzyme system. They control cell signaling and transport to the suitable subcellular compartment and are involved in the protein translocation process and in the degradation of other proteins, avoiding the eventual formation of non-functional ones [31].

HSPs identified in this study include the 10 kDa family and HSP 60 kDa family chaperone, among others. HSPs with low a molecular weight (<40 kDa) are referred to as

small HSPs and are known to perform the ‘cellular defense first line’ against stress from excessive heat and/or others, which results in protein denaturation [32]. They are present in all organisms. They form a very diverse chaperone family, which share an α -crystalline domain flanked by variable and disordered N- and C-terminal extensions, although certain structural characteristics such as an oligomeric form with a β -sandwich domain seem to remain conserved [33]. HSPs are broadly grouped according to their amino acid sequences based on the presence of certain CDs, and mainly according to their MW (kDa). They are usually grouped into six classes, which are: small HSPs, HSP100s or CLPs, HSP90s, HSP70s, HSP40s/DNAJs and chaperonins. Small HSPs protect against protein aggregation; HSP40s/DNAJs regulate the HSP70 complex; HSP70s, HSP90s and chaperonins mediate protein folding; while HSP100s/CLPs are involved in protein aggregation and solubilization [33].

Based on the documented benefits, probiotics, primarily used in food or dietary supplements, medicines and cosmetic products, are annually increasing. The global probiotic market was valued at almost USD 7 billion in 2021, with a forecast growth of 3.75% on average until 2026 having grown between 2022 and 2023 from USD 66.9 to USD 73.14 billion, with an estimated 9.3% yearly growth rate [34]. One major factor suggested for the future increasing probiotic market is the increasing consumers’ preference for healthy foods and nutritional supplements, thereby necessitating the need to ensure the all-round safety of probiotics.

It has been noted that a probiotic, as food or a supplement without a health claim or in a food or supplement with a specific health claim, must meet the criteria of being a member of a safe species in the EU [35]. It must belong to a specific probiotic strain or strains, it must be beneficial to humans or regarded as a safe microorganism based on adequate evidence and, in addition, at the end of its shelf life must deliver viable strain(s) [36]. In the present study, five genes were associated with antibiotic resistance. Probiotics are considered as safe alternatives to antibiotics and hence, should be free from antibiotic resistance, which is currently of global concern. However, being an *in silico* explorative study, there may be a need to extensively study this to validate the claim using wet-lab analyses, although *Lactobacilli* and *Enterococci* isolates have previously been reported to be resistant to cefoxitin, penicillin, vancomycin, aztreonam and oxacillin [37].

The IL-4-inducing capacity of the hypothetical proteins and their mutants as reported in this study is of importance, considering the important role IL-4 plays in immunity and its conserved nature across species. The immune system continuously communicates with the central nervous system (CNS) during health and disease [38]. The CNS plays a key role in controlling other systems of the body and may influence an organism’s general well-being. The CNS-resident macrophages and myeloid cells are usually the first cells to detect tissue damage or pathogens. These cells initiate an immune response in order to clear the tissue debris or fight the pathogen, but if the blood–brain barrier becomes permeable as a result of inflammation or trauma, immune cells that infiltrate the CNS from the periphery are pro-inflammatory within an acute inflammatory injury. However, they can also be regulatory or anti-inflammatory cells. The balance and timing of pro- and anti-inflammatory immune cells play key roles in tissue damage and repair [39,40]. T helper 2-like (TH2-like) cells and their cytokines including IL-4 inhibit TH1-like and TH17-mediated inflammatory processes, thereby serving an anti-inflammatory function [41].

The IL-4-inducing capacity of probiotics, as reported in this study, may be revealing the pathways toward the mode of mechanism of probiotics in cell immunity. Being a pleiotropic cytokine, IL-4 plays a crucial role in type 2 immunity, mainly secreted by not only TH2-like cells but also granulocytes and myeloid cells [42]. It can induce the differentiation of CD4+ T cells into TH2-like cells as well as the alternative activation of

myeloid cells (macrophages and microglia), involving morphological and transcriptional changes such as the expression of arginase 1 (Arg1), chitinase-like protein 3 (Ym1) or CD206, resulting in wound healing and the suppression of destructive inflammation [43].

IL-4 has been shown to improve immunity; enhance the functional outcome of different types of CNS injury and disease models, exerting effects on lymphocytes, microglia and neurons; as well as be recommended for therapeutic development, although with caution for consideration of possible side effects [40]. It has been reported that IL-4-stimulated Arg⁺ microglia upregulated the brain-derived neurotrophic factor and enhanced hippocampal neurogenesis in mice exposed to chronic mild stress [44]. It was reported that microglia-specific IL-4R α -deficiency suppressed neurogenesis and resulted in increased stress vulnerability. In another study involving adult zebrafish brain, IL-4 induced the phosphorylation of signal transducer and activator of transcription 6 in neural stem/progenitor cells (NSPCs) and was sufficient to restore NSPC proliferation after incubation with neurotoxic amyloid- β 42 [45]. It has been documented that IL-4-induced M2-like phenotypes to help in neuroprotection and repair [46,47], as well as was an activator of M2-like markers such as Arg1, CD206 and Ym1.

5. Conclusions

The chicken-isolated *L. reuteri* genome has one contig, with a total length of 2,085,667 bp and an average G + C content of 39.43%. It had 2471 CDS, 78 tRNA genes and 10 rRNA genes. It was associated with five antibiotic resistance genes, which were glycopeptide resistance gene cluster, vanT; chloramphenicol acetyltransferase (CAT), Erm 23S ribosomal RNA methyltransferase, tetracycline-resistant ribosomal protection protein and lincosamide nucleotidyltransferase (LNU), although none was found under the perfect RGI criterion. Hypothetical proteins of the chicken-isolated *L. reuteri* genome, labeled QHPv.2.1, QHPv.2.12 and QHPv.2.16, induced IL-4. However, QHPv.2.12 was not immunogenic. The best IL-4-inducing SVM scores were obtained by mutants from QHPv.2.1 and QHPv.2.16, followed by QHPv.2.12 and QHPv.2.5. IL-4 was upregulated in the blood, peripheral, bone marrow, ovary, tumor, lung and temporal lobe. We made attempts in this explorative study to unravel the mystery surrounding the functional mechanisms of probiotic *L. reuteri*, suggesting its sustainable applications in feed/food, cosmetic and therapeutic industries. Being an in silico explorative study, we strongly recommend wet-lab experimental validations for the claims in this study.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biologics6030021/s1>, Figure S1. Level of expression of IL-4 and IL-4R in human and mouse at different conditions: (a) scatter plot showing IL-4 and IL-4R expression (log₂ scale) in *Homo sapiens* across various anatomical parts; (b) IL-4 expression level (log₂ scale) across various neoplasms in *Homo sapiens*; (c) a heatmap showing IL-4 and IL-4R expression (linear scale) across various anatomical parts, cell types and states in *Homo sapiens*; (d) a circular plot representing the top 50 co-expressed genes with positive correlation to IL-4 across various anatomical parts, neoplasm and cell categories in *Homo sapiens*; (e) a circular plot representing the top 50 co-expressed genes with positive correlation to IL-4 across various experimental sets in *Homo sapiens*; (f) scatter plot showing IL-4 and IL-4R expression (log₂ ratio) across relevant perturbations in *Homo sapiens*; (g) boxplot showing IL-4 expression (log₂ scale) in *Mus musculus* across various cell lines; (h) a circular plot showing the top 50 co-expressed genes with positive correlation to IL-4 across various cancer categories in *Mus musculus*; (i) a heatmap showing IL-4 and IL-4RA expression (log₂ ratio) across various experimental sets in *Mus musculus*; (j) scatter plot showing IL-4 and IL-4RA expression (log₂ scale) in *Mus musculus* across various perturbations in *Mus musculus*, (k) a heatmap showing expression of cold shock proteins across various experimental set in *Rattus norvegicus* and (l) a heatmap showing expression of heat shock proteins across various experimental set in *Rattus norvegicus*.

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Abbreviations

CAT	Chloramphenicol acetyltransferase
CD	Conserved domain
CDS	Protein-coding sequences
CSP	Cold shock protein
ErmB	Erm 23S ribosomal RNA methyltransferase
GO	Gene ontology
HSP	Heat shock protein
lnu	Lincosamide nucleotidyltransferase
MP	Mutant position
MW	Molecular weight
NSPC	Neural stem/progenitor cell
pI	Isoelectric point
rRNA	Ribosomal RNA
tet(W)	Tetracycline-resistant ribosomal protection protein
tRNA	Transfer RNA

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