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Hypothetical proteins of chicken-isolated *Limosilactobacillus reuteri* subjected to in silico analyses induce IL-2 and IL-10

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Abstract

Lactic acid bacteria (LAB) probiotics are health-promoting but their characteristics, safety profile and functional mechanisms are not fully understood. Hence, this study aimed to characterize some hypothetical proteins of the chicken-isolated *Limosilactobacillus reuteri* genome and unravel their IL-2 and IL-10-inducing potential to understand mechanisms of their immunological functionality for sustainable applications. The selected proteins were subjected to in silico analyses for transmembrane topology, sub-cellular localization, IL-2 and IL-10-inducing ability and IL-2 and IL-10 gene expression across various conditions. IL-2 and IL-10-inducing mutants were statistically analyzed using a one-way analysis of variance of a general linear model of SAS and statistical significance was set at $p < 0.05$. The analyzed proteins are stable under a wide temperature range. All the hypothetical proteins are IL-2 and IL-10-inducing but QHPv.2.12, QHPv.2.13 and QHPv.2.15 are non-immunogenic. The evaluated mutants are IL-2 and IL-10-inducers but QHPv.2.13 and QHPv.2.15 are not immunogenic. This study sheds light on understanding the functional mechanisms of chicken-isolated *L. reuteri* and suggests it or its proteins as potent candidates for feed additive and therapeutic purposes.

Keywords Animal nutrition, Bacteria, Cancer, Gene expression, Human nutrition, Probiotics

Introduction

The intensive system of animal production has many benefits but also exposes animals to stress [1], which may negatively affect their immune functions, leading to pathogenic colonization [2]. This may cause a serious health implication to the animals and consumers of such animal products, considering the potential of disease outbreaks which may lead to huge economic losses. It is therefore imperative to find alternative feed additives that can effectively control pathogenic invasion, promote growth and help address the issues mentioned above. Probiotics are important alternatives that have been used in human and animal nutrition, for cosmetic and therapeutic purposes, which are living microorganisms that when consumed in adequate amounts offer health benefits to the host(s).

Probiotics and beneficial microorganisms help develop immunity against pathogens, ensure growth and improve animal and human health [3, 4]. Probiotics have been suggested as being safe and cost-effective for animal production [5]. They have been shown to improve immune responses, and growth performance, neutralize various enterotoxins [6], enhance animal food safety and reduce the risk of gastrointestinal colonization by foodborne pathogens [7].

A major source of probiotics used in human and animal nutrition is the Lactic acid bacteria (LAB) with accompanying benefits including enhancing the host's health, anti-inflammatory, gut microbiota modulation,

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immunomodulation and antimicrobial effects [8]. LAB produce lactic acid, antioxidants, short-chain fatty acids and bacteriocins, which inhibit bacterial pathogenic growth [9]. They play major roles in influencing the host's intestinal microbial balance, thereby contributing to innate intestinal immunity and homeostasis regulation [10].

Lactobacillus, the major candidate strain introduced for probiotic purposes accommodates more than 200 species [11] and has been reported to improve digestion of feed and nutrient absorption [2]. A recent study [12] reported that the five strains of *Lactobacillus* including *L. reuteri, L. ingluviei*, and *L. acidophilus* isolated from the chicken gut have both probiotic and cholesterol removal abilities. Another recent study [13] revealed that 12 bacteria from selected free-range indigenous Ethiopian chicken gastrointestinal tract possess probiotic ability. A lot is known about the health benefits of LAB probiotics but not all LAB are probiotics, and there remains a lot to be understood about their characteristics, safety profile and functional mechanisms. Understanding their uncharacterized or hypothetical proteins may shed light on their characteristics, safety profile and functional mechanisms. This study was therefore, carried out to characterize some uncharacterized proteins of chicken-isolated *Limosilactobacillus reuteri* genome as well as unravel their potential to induce IL-2 and IL-10 to understand mechanisms of their immunological functionality for sustainable applications, perhaps in animal and human nutrition and for cosmetic and therapeutic purposes.

Results

The results presented in this section were generated through in silico analyses, suggesting the need for wet lab validation. Multiple sequence alignment was done for sequence similarity check for the selected hypothetical proteins of the chicken-isolated *Limosilactobacillus reuteri*. The phylogenetic tree connecting the evaluated hypothetical proteins is presented in Fig. 1 in both radial (a) and tree (b) forms showing their relationship with one another.

The result of the transmembrane topology and subcellular localization prediction of the selected proteins is presented in Table 1. Of the evaluated proteins, all heat and cold shock proteins are globular proteins except HSPHtpX. QHPv.2.4 is signal peptide, QHPv.2.5 is transmembrane with signal peptide, QHPv.2.8, QHPv.2.11, QHPv.2.14 and QHPv.2.15 are transmembrane while the remaining hypothetical proteins are globular. The sub-cellular localization result revealed that all the evaluated heat and cold shock proteins of the chicken-isolated *Limosilactobacillus reuteri* are found in the cytoplasm except for HSPHtpX which is found in the cytoplasmic membrane. The sub-cellular localizations of QHPv.2.2, QHPv.2.3, QHPv.2.4, QHPv.2.6, QHPv.2.7, QHPv.2.9, QHPv.2.12, QHPv.2.13 and QHPv.2.17 are unknown, QHPv.2.16 is extracellular while the remaining ones are found in the cytoplasmic membrane.

The amino acid composition of the evaluated proteins is presented in Fig. 2. The predominant amino acids (AAs) in CSP1 are Gly, Glu and Val (Fig. 2a). Val, Glu, Lys and Gly are the predominant AA of HSP10kda (Fig. 2b). For HSP60kda, the predominant AA are Ala, Val and Gly (Fig. 2c). Lys, Asp, Val and Ala are the predominant AA of HSPGrpE (Fig. 2d). Ala and Leu are the predominant AA of HSPHtpX (Fig. 2e). The predominant AA of CSP2 are Val, Gly and Glu (Fig. 2f). The predominant AA for QHPv.2.1 are Lys, Gly, Ile and Ser (Fig. 2g) and for QHPv.2.2 the predominant AA are Ile, Ala and Asp (Fig. 2h). Lys and Asp are the most frequent AAs of QHPv.2.3 (Fig. 2i) while Leu, Ala, Gly and Ser are the most frequent AA of QHPv.2.4 (Fig. 2j). The major AAs of QHPv.2.5 are Leu, Ile and Trp (Fig. 2k) while the main AA for QHPv.2.6 are Lys, Leu, Gly and Ile (Fig. 2l). Leu, Glu, Asp and Lys are the major AA of QHPv.2.7 (Fig. 2m) and the major AA of QHPv.2.8 are Phe, Leu and Arg (Fig. 2n). The major AA of QHPv.2.9 are Lys, Glu, Asp and Met (Fig. 2o) and the major AA of QHPv.2.11 are Leu, Phe and Ala (Fig. 2p). The predominant AA of QHPv.2.12 are Asn, Thr, Glu, Leu and Val (Fig. 2q); the most common AA of QHPv.2.13 are Leu and Ser (Fig. 2r); for QHPv.2.14, the main AA are Leu, Ile, Val and Met (Fig. 2s); Gly, Ile, Val, Phe and Leu are the major AA of QHPv.2.15 (Fig. 2t); Glu, Lys, Ile and Ser are the major AA of QHPv.2.16 (Fig. 2u) and the predominant AA of QHPv.2.17 are Ile, Ala and Asp (Fig. 2v).

The physicochemical properties of selected proteins of the chicken-isolated *Limosilactobacillus reuteri* genome are presented in Table 2. The amino acid length of the proteins ranges between 29 (QHPv.2.7) and 543 (HSPGrpE). Molecular weight (MW) ranges between 3382.18 (QHPv.2.6) and 59,030.05 (HSPGrpE), pI range between 4.14 (QHPv.2.12) and 10.71 (QHPv.2.6), the total negatively charged residue range between 0 (QHPv.2.15) and 89 (HSPGrpE) and the total positively charged residue range between 1 (QHPv.2.5) and 72 (HSPGrpE). HSPGrpE has the highest total number of atoms (8377) while QHPv.2.7 has the lowest total number of atoms. HSPHtpx, CSP2, QHPv.2.1, QHPv.2.5, QHPv.2.8, QHPv.2.9 and QHPv.2.16 are unstable in nature. Aliphatic indices are generally high except for CSP1 (58.94) and QHPv.2.2 (43.33). GRAVY indices are low for all the proteins except for QHPv.2.5 (1.550), QHPv.2.11 (1.378), QHPv.2.14 (1.782) and QHPv.2.15 (1.635). The three-dimensional structures of the evaluated proteins of the

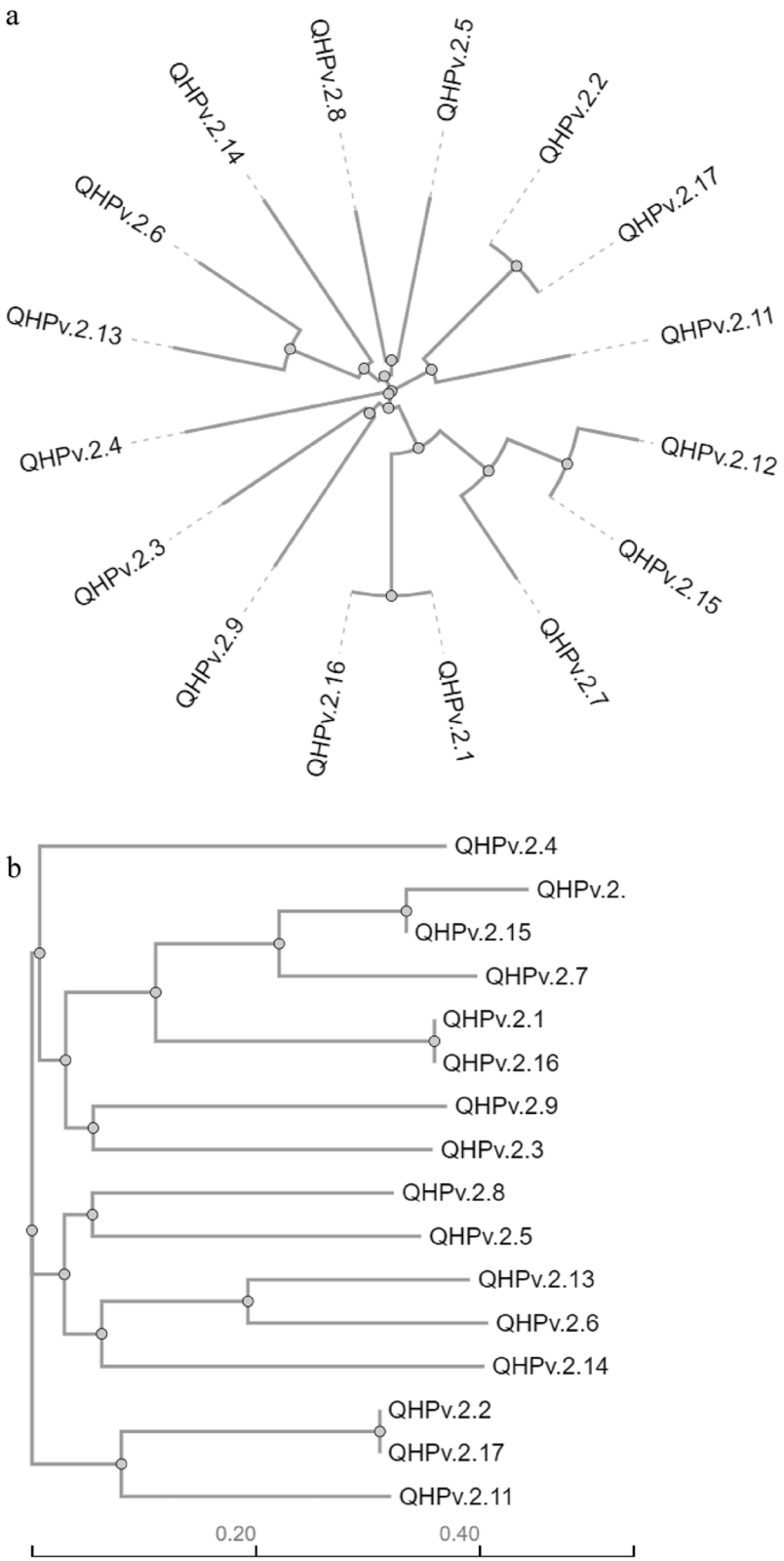


Fig. 1 Phylogenetic tree of the hypothetical proteins of chicken-isolated *L. reuteri* genome generated from multiple sequence alignment of their sequence: **a** radial view of the hypothetical proteins **b** tree view of the hypothetical proteins

Table 1 Topology and sub-cellular localization prediction of selected proteins of chicken-isolated *Limosilactobacillus reuteri* genome

Proteins	Topology	Sub-cellular localization
CSP1	Globular	Cytoplasmic
HSP10kda	Globular	Cytoplasmic
HSP60kda	Globular	Cytoplasmic
HSPGrpE	Globular	Cytoplasmic
HSPHtpX	Transmembrane	Cytoplasmic Membrane
CSP2	Globular	Cytoplasmic
QHPv.2.1	Globular	Extracellular
QHPv.2.2	Globular	Unknown
QHPv.2.3	Globular	Unknown
QHPv.2.4	Signal peptide	Unknown
QHPv.2.5	Transmembrane with signal peptide	Cytoplasmic Membrane
QHPv.2.6	Globular	Unknown
QHPv.2.7	Globular	Unknown
QHPv.2.8	Transmembrane	Cytoplasmic Membrane
QHPv.2.9	Globular	Unknown
QHPv.2.11	Transmembrane	Cytoplasmic Membrane
QHPv.2.12	Globular	Unknown
QHPv.2.13	Globular	Unknown
QHPv.2.14	Transmembrane	Cytoplasmic Membrane
QHPv.2.15	Transmembrane	Cytoplasmic Membrane
QHPv.2.16	Globular	Extracellular
QHPv.2.17	Globular	Unknown

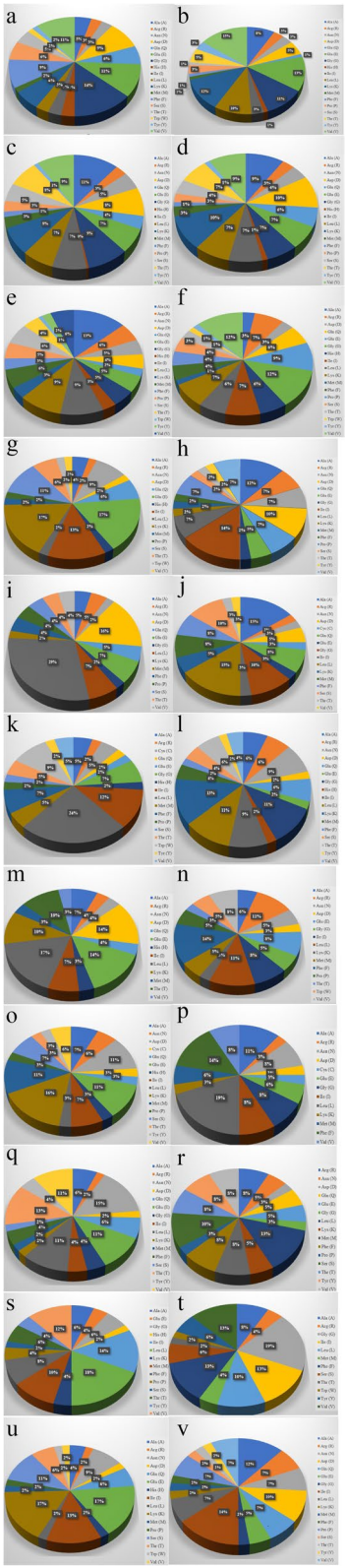
chicken-isolated *Limosilactobacillus reuteri* genome are presented in Fig. 3.

Figure 4 shows the IL-10 prediction by selected hypothetical proteins of chicken-isolated *Limosilactobacillus reuteri* when the nonimmunogenic but IL-10-inducing proteins (QHPv.2.12, QHPv.2.13 and QHPv.2.15) have been removed; a. according to their SVM values, all the selected proteins induce IL-10 but not all are immunogenic. b. According to immunogenicity values, QHPv.2.8 has the highest immunogenicity value, being followed by QHPv.2.7 and QHPv.2.5 has the lowest value, c. IL-2 prediction by selected hypothetical proteins of the chicken-isolated *Limosilactobacillus reuteri,* when the IL-2-inducing but nonimmunogenic proteins (QHPv.2.12, QHPv.2.13 and QHPv.2.15) have been removed according to their ML values; d. when one of the nonimmunogenic (QHPv.2.15) is included to show the trend. The evaluated proteins induce IL-2 but not all are immunogenic.

The statistical analysis result of immunogenic IL-2 and IL-10 inducing mutants of hypothetical proteins of chicken-isolated *Limosilactobacillus reuteri* is presented in Table 3. All the selected hypothetical proteins induce IL10 and IL-2 but not all are immunogenic. Only the immunogenic IL-10 and IL-2 inducers are presented in Table 3. According to SVM values, HPv.2.8 (1.7651) has the highest value, followed by QHPv.2.14 (1.3928) and QHPv.2.11 (1.2590). The lowest value is obtained by QHPv.2.3 (0.4093) and QHPv.2.9 (0.3342), which are significantly ($p < 0.05$) lower compared to other mutant categories. QHPv.2.1 is statistically similar to QHPv.2.7 and QHPv.2.16, while QHPv.2.4, QHPv.2.7 and QHPv.2.12 are statistically similar. ML value is significantly ($p < 0.05$) higher for QHPv.2.1 (0.8762), QHPv.2.5 (0.8742), QHPv.2.6 (0.8562) and QHPv.2.16 (0.8760), followed by QHPv.2.4 (0.7547), QHPv.2.8 (0.7522) and QHPv.2.14 (0.7710), while QHPv.2.9 (0.5795) obtained the least value. The MW of the proteins ranges between 3382.44 (QHPv.2.7) and 5636.26 (QHPv.2.14). QHPv.2.6 has the highest isoelectric point (pI) (10.618), followed by QHPv.2.3 (9.681). QHPv.2.2, QHPv.2.7, QHPv.2.9, QHPv.2.11 and QHPv.2.17 are statistically similar. QHPv.2.12 (4.224) has the lowest pI, which is statistically similar to the values obtained for QHPv.2.2, QHPv.2.7 and QHPv.2.17.

The top two immunogenic IL-2 and IL-10 inducing mutants of hypothetical proteins of chicken-isolated *Limosilactobacillus reuteri* selected based on their SVM (ML) and immunogenicity scores are presented in Table 4 with their corresponding mutant positions, pI and molecular weight. A scatter plot showing IL-2 and IL-10 expression (log2 ratio) in *Mus musculus* according to anatomical parts and cell types is presented in Fig. 5a. IL-10 is up-regulated in the spleen (CD4 T-cell) and gonadal adipose tissue (macrophage), but down-regulated in most anatomical parts and cell types. IL-2 expression level is mostly average.

Figure 5b shows a circular view of positively co-expressed genes with IL-10 in *Mus musculus* across various anatomical parts, neoplasm and cell categories. At the top of the list of co-expressed genes are: RIKEN cDNA 5430437J10 gene (5430437J10Rik), tumor necrosis factor (Tnf), interleukin 12b (Il12b), taste receptor, type 2, member 105 (Tas2r105), CD209e antigen (Cd209e), vomeronasal 1 receptor 46 (Vmn1r46) and chemokine (C–C motif) receptor (Ccr3). A heatmap showing IL-2 and IL-10 expression (Log2 ratio) across various experimental sets in *Homo sapiens* is presented in Fig. 5c. T-cell activation study 1 / quiescent CD4 + T-cell sample, T-cell activation study 3 / resting CD4 T-lymphocyte (crude fraction) sample, T-helper activation study 1 (180 min) / unstimulated helper T-cell sample, T-helper activation study 1 (160 min) / unstimulated helper T-cell sample, T-helper activation study 1 (260 min) / unstimulated helper T-cell sample, T-helper activation study 1 (140 min) / unstimulated helper T-cell sample, T-helper activation study 1



◄ **Fig. 2** Amino acid (AA) composition of the evaluated proteins of chicken-isolated *Limosilactobacillus reuteri* genome: **a** AA composition for CSP1, **b** AA composition for HSP10kda, **c** AA composition for HSP60kda, **d** AA composition for HSPGrpE, **e** AA composition for HSPHtpX, **f** AA composition for CSP2, **g** AA composition for QHPv.2.1, **h** AA composition for QHPv.2.2, **i** AA composition for QHPv.2.3, **j** AA composition for QHPv.2.4, **k** AA composition for QHPv.2.5, **l** AA composition for QHPv.2.6, **m** AA composition for QHPv.2.7, **n** AA composition for QHPv.2.8, **o** AA composition for QHPv.2.9, **p** AA composition for QHPv.2.11, **q** AA composition for QHPv.2.12, **r** AA composition for QHPv.2.13, **s** AA composition for QHPv.2.14, **t** AA composition for QHPv.2.15, **u** AA composition for QHPv.2.16 and **v** AA composition for QHPv.2.17

(220 min) / unstimulated helper T-cell sample, T-helper activation study 1 (200 min) / unstimulated helper T-cell sample, T-helper activation study 1 (240 min) / unstimulated helper T-cell sample, T-helper activation study 1 (120 min) / unstimulated helper T-cell sample, T-helper activation study 1 (280 min) / unstimulated helper T-cell sample, T-helper activation study 1 (300 min) / unstimulated helper T-cell sample, T-helper activation study 1 (340 min) / unstimulated helper T-cell sample and PMA; ionomycine study 2 / unstimulated CD4 memory T-cell sample resulted in up-regulation of both IL-2 and IL-10. CAR T cell study 4 (PSCA-28t28Z; post-infusion) / CAR T cell study resulted in down-regulation of IL-2.

A heatmap showing IL-2 and IL-10 expression levels (linear scale) across various cancer categories in *Homo sapiens* is presented in Fig. 5d. MGUS; (extranodal) marginal zone B-cell lymphoma, NOS malignant lymphoma, follicular, NOS chronic lymphocytic B-cell leukemia Hodgkin disease, NOS tongue, squamous cell carcinoma, NOS and sigmoid colon, adenoma, NOS, unstated behaviour resulted in up-regulated IL-2. Anaplastic large cell lymphoma, T-cell and Null cell type and prostate, neoplasm, malignant, stroma up-regulated IL-10 in *Homo sapiens*.

A circular view of positively co-expressed genes with IL-2 across anatomical parts, neoplasm and cell types in *Homo sapiens* is shown in Fig. 5e. At the very top of the list are: long intergenic non-protein coding RNA 1692 (LINC01692), novel transcript (AC087516.1, AC007686.2, AC012361.1), long intergenic non-protein coding RNA 2619 (LINC02619), long intergenic non-protein coding RNA 2318 (LINC02318), tec (AC026422.1), long intergenic non-protein coding RNA 2237 (LINC02237), long intergenic non-protein coding RNA 2465 (LINC02465) and ubiquitin D pseudogene 1 (UBDP1).

Table 2 Physicochemical properties of selected proteins of chicken-isolated *Limosilactobacillus reuteri* genome

Proteins	AA	MW	pI	-R	+R	Formular	# atoms	EC (M^{-1} cm^{-1})	II	AI	GRAVY
CSP1	66	7340.98	4.49	13	6	$C_{324}H_{483}N_{87}O_{107}S$	1002	6990	15.48	58.94	-0.583
HSP10kda	94	10,165.67	5.20	17	14	$C_{452}H_{746}N_{120}O_{142}S$	1461	4470*	11.87	101.49	-0.261
HSP60kda	542	57,097.72	4.71	81	58	$C_{2467}H_{4091}N_{683}O_{827}S_{17}$	8085	8940*	30.10	94.13	-0.171
HSPGrpE	543	59,030.05	5.07	89	72	$C_{2555}H_{4245}N_{715}O_{846}S_{16}$	8377	10,430*	34.67	92.12	-0.420
HSPHtpX	298	32,765.22	6.76	28	27	$C_{1465}H_{2349}N_{401}O_{415}S_{17}$	4647	26,930	46.63	104.23	0.196
CSP2	67	7592.76	9.30	8	10	$C_{342}H_{544}N_{100}O_{94}S$	1081	6990*	48.53	90.00	-0.382
QHPv.2.1	47	5512.19	6.56	9	9	$C_{236}H_{394}N_{68}O_{81}S$	780	5500	87.36	68.51	-1.340
QHPv.2.2	42	4709.40	4.53	6	4	$C_{208}H_{340}N_{58}O_{64}S$	671	1490*	26.40	116.19	-0.064
QHPv.2.3	45	5293.08	9.65	10	15	$C_{230}H_{389}N_{67}O_{73}S$	760	**	31.73	43.33	-1.798
QHPv.2.4	40	4221.93	5.87	3	3	$C_{184}H_{299}N_{49}O_{56}S_4$	592	0*	33.21	90.50	0.415
QHPv.2.5	42	4982.19	6.49	1	1	$C_{246}H_{368}N_{52}O_{50}S_4$	720	$23,615^{+}$, $23,490^{++}$	53.53	157.86	1.550
QHPv.2.6	47	5243.18	10.71	2	9	$C_{229}H_{391}N_{71}O_{65}S_2$	758	1490*	16.70	93.40	-0.451
QHPv.2.7	29	3382.83	4.50	8	4	$C_{145}H_{245}N_{39}O_{51}S$	481	**	23.87	111.03	-0.648
QHPv.2.8	37	4513.39	8.49	4	5	$C_{215}H_{324}N_{54}O_{49}S_2$	644	11,000	52.46	102.70	0.359
QHPv.2.9	38	4372.04	5.08	8	6	$C_{184}H_{305}N_{49}O_{63}S_5$	606	1490^{*+}, 1490^{++}	42.96	64.21	-0.787
QHPv.2.11	36	4003.88	4.68	3	2	$C_{189}H_{297}N_{43}O_{46}S_3$	578	0^{*+}, 0^{++}	16.84	143.61	1.378
QHPv.2.12	47	5306.89	4.14	6	2	$C_{232}H_{367}N_{61}O_{79}S$	740	2980*	18.33	95.32	-0.232
QHPv.2.13	38	4594.34	9.40	3	5	$C_{207}H_{323}N_{53}O_{59}S_3$	645	4470*	39.71	74.21	-0.297
QHPv.2.14	50	5615.12	6.51	2	2	$C_{273}H_{440}N_{54}O_{61}S_5$	833	2980*	12.90	165.60	1.782
QHPv.2.15	48	5151.25	9.69	0	2	$C_{253}H_{379}N_{55}O_{56}S_2$	745	9970	28.53	133.96	1.635
QHPv.2.16	47	5512.19	6.56	9	9	$C_{236}H_{394}N_{68}O_{81}S$	780	5500	87.36	68.51	-1.340
QHPv.2.17	42	4709.40	4.53	6	4	$C_{208}H_{340}N_{58}O_{64}S$	671	1490*	26.40	116.19	-0.064

MW molecular weight, *-R* total negatively charged residue (Asp + Glu), *+R* total positively charged residue (Arg + Lys), *#atoms* total number of atoms, *II* instability index, *AI* aliphatic index; *This protein does not contain any Trp residues; EC = extinction coefficient; $^{+}$assuming all pairs of Cys residues form cystines; $^{++}$assuming all Cys residues are reduced; ** As there are no Trp, Tyr or Cys in the region considered, this protein is not be visible by UV spectrophotometry

Discussion

Bioinformatics tools were used to generate results for parameters such as physicochemical properties, structures and IL-2 and IL-10-inducing potential. Physicochemical properties of a peptide or protein are used to characterize such peptide or protein using different parameters. The extinction coefficient tells how much light a protein absorbs at a certain wavelength. The half-life predicts the time it takes for half of the amount of protein in a cell to disappear after its synthesis in the cell. In the present study, the half-life estimation is the same for all the evaluated proteins (30 h (mammalian reticulocytes, in vitro), > 20 h (yeast, in vivo) and > 10 h (*Escherichia coli*, in vivo). This result relies on the "N-end rule", which relates the half-life of a protein to the identity of its N-terminal residue. This result is for three model organisms: humans, yeast and *E. coli* [14]. The instability indicates the stability of evaluated proteins in a test tube, a protein with an instability index smaller than 40 is predicted as stable, while a protein with a value above 40 is unstable [14, 15].

In the present study, HSPHtpx, CSP2, QHPv.2.1, QHPv.2.5, QHPv.2.8, QHPv.2.9 and QHPv.2.16 have values above 40, hence are unstable. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains, that is alanine, valine, isoleucine, and leucine, and may be regarded as a positive factor for the increase of thermostability of globular proteins [14, 16].

The GRAVY value for a protein or a peptide is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence [14, 17]. High aliphatic indices in this study indicate thermostability under wide temperature ranges. The GRAVY values usually range between -2 and + 2. A negative value implies hydrophilicity while a positive value indicates hydrophobicity [17]. In the present study, HSPHtpX, QHPv.2.4, QHPv.2.5, QHPv.2.8, QHPv.2.11, QHPv.2.14 and QHPv.2.15 have positive values, indicating hydrophobicity. Higher negative GRAVY values indicate that the proteins are hydrophilic in nature with good solubility and vice-versa. pI is the pH at which proteins carry no charge or when the total negative and positive charges are equal, indicating whether the protein is acidic or basic. In the present study, CSP2, QHPv.2.3, QHPv.2.6, QHPv.2.8, QHPv.2.13 and QHPv.2.15 have pI greater than 7 indicating that they are basic in nature,

Human IL-2 is a pleiotropic cytokine that plays crucial roles in immune responses [18], promoting the

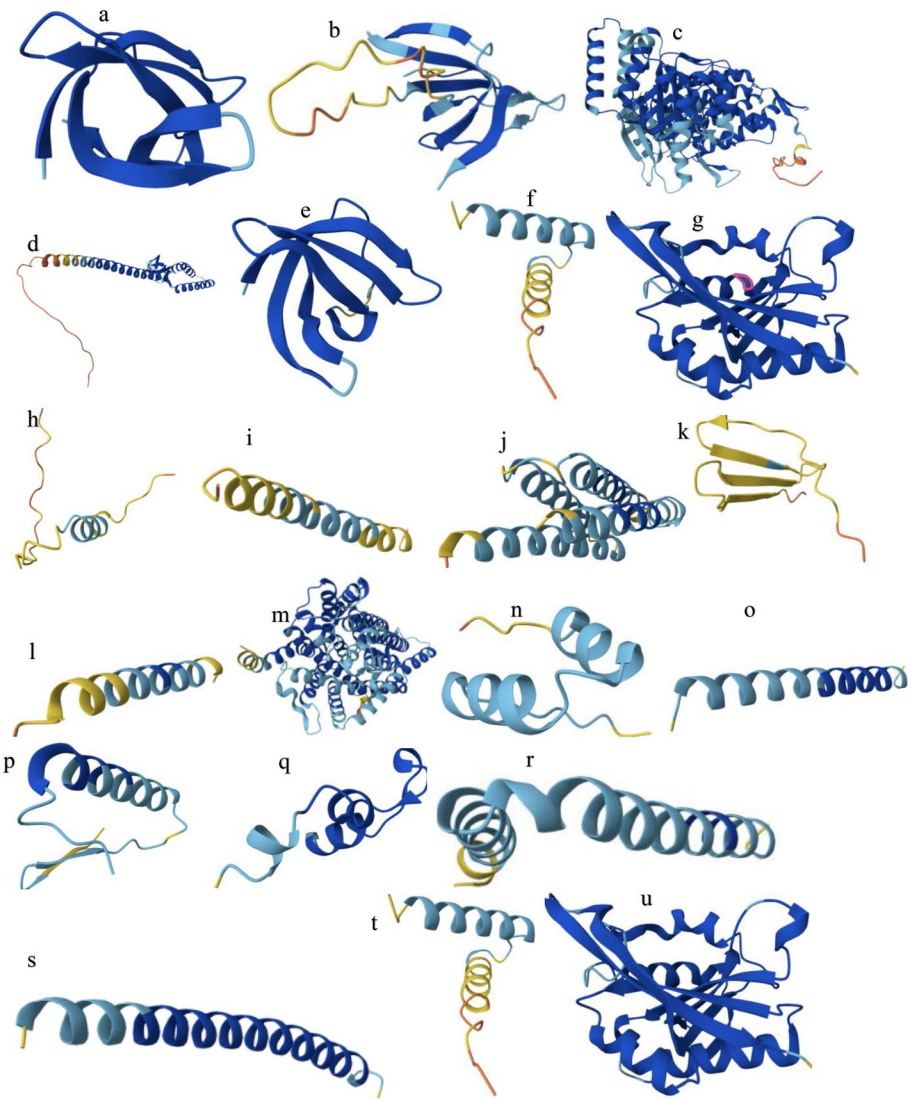
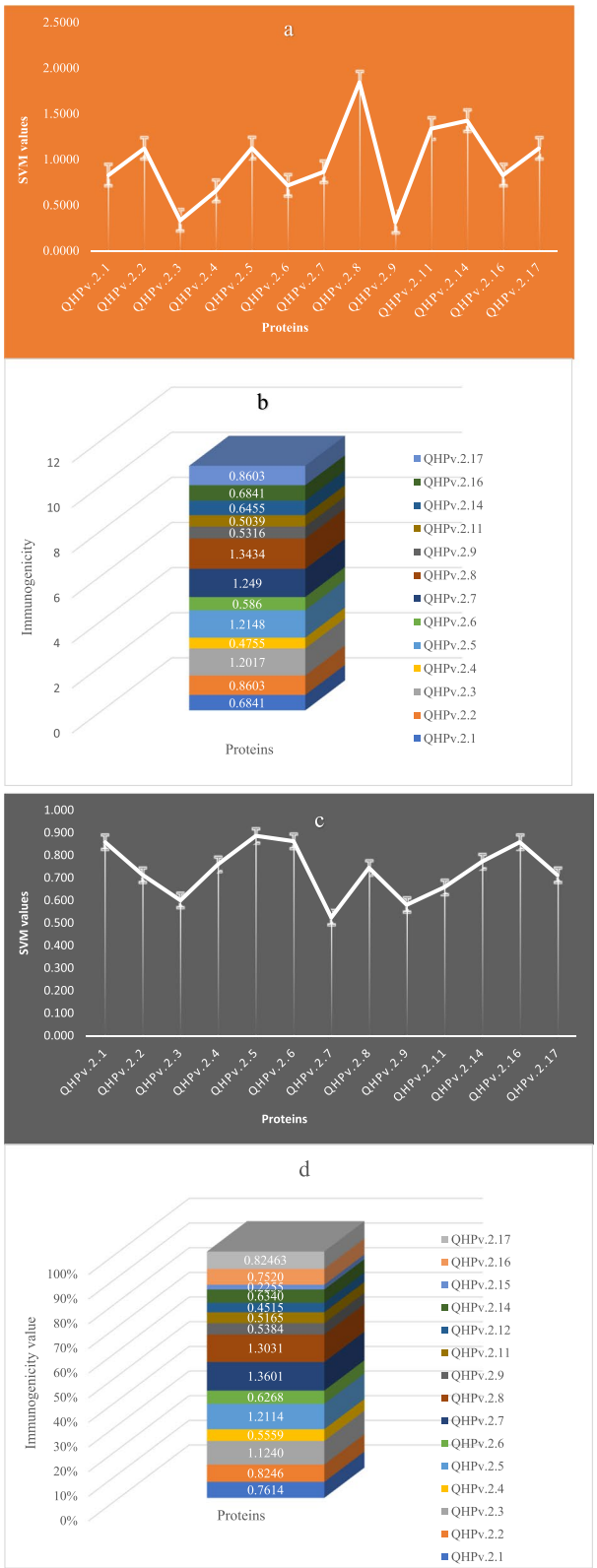


Fig. 3 Three-dimensional structures of evaluated proteins of chicken-isolated *Limosilactobacillus reuteri* genome as obtained from Alphafold 2.0 for: **a** CSP1, **b** HSP10kda, **c** HSP60kda, **d** HSPGrpE, **e** CSP2, **f** QHPv.2.1, **g** QHPv.2.2, **h** QHPv.2.3, **i** QHPv.2.4, **j** QHPv.2.5, **k** QHPv.2.6, **l** QHPv.2.7, **m** QHPv.2.8, **n** QHPv.2.9, **o** QHPv.2.11, **p** QHPv.2.12, **q** QHPv.2.13, **r** QHPv.2.14, **s** QHPv.2.15, **t** QHPv.2.16 and **u** QHPv.2.17

differentiation, proliferation and survival of T and B cells, as well as enhancing the cytolytic activity of natural killer (NK) cells in the innate immune defense [19]. It is a glycoprotein mainly produced by activated CD4 + and CD8 + T lymphocytes [20], binding to a heterotrimeric receptor on the surface of immune cells to exert its effects. The high affinity IL-2 receptor consists of three separate, noncovalently linked subunits, known as IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγc (CD132) [21]. The IL-2Rα chain captures IL-2 at the cell surface and delivers it to IL-2Rβγc chains, as the signaling region of the receptor [21]. The β and γc chains are expressed on T cells while the α chain expression is restricted to early thymocytes, Tregs and activated T cells. Both β and γc chains form an IL-2 receptor (IL-2R) with intermediate affinity. The α chain cannot form a functional receptor [21] when βγc chains are lacking.

IL-2 is the first cytokine whose encoding gene was cloned and sequenced [22]. Being regarded as a potent stimulator of immune responses, it was used in clinical trials of IL-2 in patients with metastatic cancer [23], but because of its very short half-life, large doses had to be given in order to achieve a biologically effective level, which eventually stimulated the production of many other cytokines, resulting in severe toxicities, mainly from vascular leakage, thereby limiting its application as



◀ **Fig. 4** IL-2 and IL-10 prediction by selected hypothetical proteins of chicken-isolated *Limosilactobacillus reuteri* genome when the IL-10 inducing but nonimmunogenic proteins (QHPv.2.12, QHPv.2.13 and QHPv.2.15) have been removed; **a** according to their SVM values, **b** according to immunogenicity values, **c** IL-2 prediction by selected hypothetical proteins of chicken-isolated *Limosilactobacillus reuteri,* when the IL-2 inducing but nonimmunogenic proteins have been removed according to their ML values; **d** when one of the nonimmunogenic (QHPv.2.15) is included to show the trend

a first-line or adjunct cancer therapy [24, 25]. However, studies are ongoing to overcome this limitation [26]. IL-2 was also used to treat lymphomas and leukemias [27].

IL-2 or IL-2 receptor a or b chain absence in mice resulted in massive lymphadenopathy and splenomegaly and manifestations of autoimmune diseases, such as colitis and autoimmune hemolytic anemia [28–30]. Also, a brief treatment of mice with antibodies that neutralize IL-2 or block the IL-2 receptor led to acute autoimmune manifestations [31, 32], the findings which were complemented by human studies showing that T cells in patients with lupus and other autoimmune diseases produce reduced amounts of IL-2 [33], indicating that a major and essential function of IL-2 is to maintain self-tolerance and prevent autoimmunity and that the depletion or decreased production of this cytokine is associated with systemic autoimmunity [25]. Low-dose IL-2 was adjudged safe and effective in treating vasculitis and chronic graft-versus-host disease [34, 35].

IL-10 is a pleiotropic cytokine with anti-inflammatory and pro-inflammatory properties. Its dual functions have been attributed to its producer, the target cell, the available IL-10 levels and the microenvironmental co-factors including other cytokines [36]. IL-10 ensures immune homeostasis and prevents immune-mediated inflammatory diseases and exuberant immune responses to pathogens [37]. Several innate and adaptive immune systems produce and respond to IL-10 [40]. IL-10's immune regulation is a phylogenetic ancient mechanism to induce tolerance and fine-tune the inflammatory response, found in amphibians, fish, mammals and all vertebrates [38].

It has been shown that IL-10 is a key molecule displaying suppressive activity against effector T cells in the intestine and plays a key role in maintaining intestinal homeostasis [39]. Deleting IL-10 receptors in T helper 2 (TH2) cells was reported to exacerbate TH2-cell-driven airway inflammation [40]. T helper 1 (TH1) cells are other sources of IL-10 [41] and CD4 + T cells co-producing IFN-γ and IL-10 have been observed in various bacterial, parasitic and viral infection models (reviewed in [42, 43]. Foxp3 + regulatory T cells (Treg) are another important source of IL-10 in multiple organs such as the skin, intestine, lung, and central nervous system, controlling

Table 3 Immunogenic IL-2 and IL-10 inducing mutants of evaluated hypothetical proteins

Mutant source	SVM value (IL10)	MW	pI	ML value (IL-2)
QHPv.2.1	0.8502^{e}	5504.04^{b}	7.878^{c}	0.8762^{a}
QHPv.2.2	1.0947^{d}	4716.16^{f}	4.463^{fg}	0.7200^{c}
QHPv.2.3	0.4093^{h}	5305.17^{c}	9.681^{b}	0.6130^{e}
QHPv.2.4	0.6926^{fg}	4237.66^{i}	6.659^{e}	0.7547^{b}
QHPv.2.5	1.1315^{d}	5002.09^{e}	6.839^{de}	0.8742^{a}
QHPv.2.6	0.6310^{g}	5224.17^{d}	10.618^{a}	0.8562^{a}
QHPv.2.7	0.7809^{ef}	3382.44^{k}	4.416^{fg}	0.5278^{g}
QHPv.2.8	1.7651^{a}	4515.25^{g}	8.260^{c}	0.7522^{b}
QHPv.2.9	0.3342^{h}	4398.01^{h}	5.115^{f}	0.5795^{f}
QHPv.2.11	1.2590^{c}	4018.36^{j}	5.095^{f}	0.6626^{d}
QHPv.2.12	0.7238^{fg}	5303.98^{c}	4.224^{g}	0.6272^{e}
QHPv.2.14	1.3928^{b}	5636.26^{a}	7.496^{cd}	0.7710^{b}
QHPv.2.16	0.8436^{e}	5498.93^{b}	7.597^{cd}	0.8760^{a}
QHPv.2.17	1.0947^{d}	4716.66^{f}	4.463^{fg}	0.7200^{c}
SEM	0.0314	50.83	0.19	0.0092

MW Molecular weight, *pI* isoelectric point, *SEM* pooled standard error of the mean

$^{abc\cdots}$ Means with different superscripts within the same columns are significantly ($p < 0.05$) different

immune homeostasis and inflammation via the production of IL-10 [44, 45]. Mechanistically, IL-10 produced by Foxp3 + Treg promotes their activity via a positive feedback loop and suppresses the pro-inflammatory function of antigen-presenting cells (APCs) and TH17 cells.

IL-10 is an important cytokine produced by T regulatory type 1 cells (TR1) and regulatory T cell subsets (Foxp3 + Treg). IL-10 sensing is a crucial signal, for maintaining their phenotype and roles [44]. It has been observed in *Homo sapiens* that the loss of function mutations and single-nucleotide polymorphisms in the IL-10R subunits or IL-10 were associated with immune-mediated inflammatory diseases, typically inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA), which is an indication of the crucial immune regulatory role of IL-10 [46, 47]. IL-10 can function in pro-inflammatory processes in CD4 + T cells and promote the killing capacity and memory formation in CD8 + Tcells [48] as well as promoting B-cell survival, proliferation and the capacity to produce antibodies [49], which may benefit the humoral response to foreign antigens or drive pathology in SLE [50].

A mouse model of transfer colitis in which macrophages could not sense IL-10 was found to develop spontaneous colitis and was inadequate in controlling the T helper 17 (TH17) cell response [51]. During the resolution of inflammation, IL-10 signaling in macrophages was found to promote the phagocytosis of apoptotic cells in mouse models of peritonitis, LPS-induced lung inflammation, and arteriosclerosis [45, 52]. Considering its dual roles, IL-10 has been evaluated for treating autoimmune diseases and cancer. IL-10 − / − mice developed spontaneous colitis, and mutations and single nucleotide polymorphisms (SNPs) in IL10 and IL10RA/IL10RB genes were associated with the development of juvenile and adult IBD in humans [46, 53], suggesting IL-10 as a potent candidate for IBD treatment.

The function of IL-10 signaling during inflammation may be summarized thus: suppression of pro-inflammatory TH17 cell responses in autoimmunity by IL-10 produced by Foxp3 + Treg and TR1 cells [54, 55]; IL-10 promotes the exacerbation of immune responses via the induction of B cell proliferation, survival and antibody production during autoimmunity [56]; and reduction of the antigen-sensitivity of CD8 + T cells by IL 10 via induction of N-glycan branching on glycoproteins including the T cell receptor during chronic viral infection [57]. IL-10 is an important mediator of host adaptive and innate immunity with a multifaceted nature in stimulating or inhibiting important immune pathways. As an immune modulator, IL-10 can decrease detrimental inflammation, inhibit cancer progression, and curb disease conditions including being suggested as playing an important role in the acute SARS-CoV 2 infection phase and the post-infection period [58]. The author strongly recommends wet lab validations of the results presented in this article, as that may give wholistic understanding of the results.

Conclusion

Selected proteins of the chicken-isolated *L. reuteri,* genome were explored for their IL-2 and IL-10-inducing potential using in silico analyses. The proteins are stable in nature except for HSPHtpx, CSP2, QHPv.2.1, QHPv.2.5, QHPv.2.8, QHPv.2.9 and QHPv.2.16. The sub-cellular localization of QHPv.2.2, QHPv.2.3, QHPv.2.4, QHPv.2.6, QHPv.2.7, QHPv.2.9, QHPv.2.12, QHPv.2.13 and QHPv.2.17 is unknown, QHPv.2.16 is extracellular while the remaining ones are found in the cytoplasmic membrane. CSP2, QHPv.2.3, QHPv.2.6, QHPv.2.8, QHPv.2.13 and QHPv.2.15 are basic in nature. The evaluated proteins are stable under a wide temperature range. All the hypothetical proteins induce IL-2 and IL-10 but QHPv.2.12, QHPv.2.13 and QHPv.2.15 are non-immunogenic. All the hypothetical mutant proteins are IL-2 and IL-10-inducers but QHPv.2.13 and QHPv.2.15 are not immunogenic. Findings from this study provide insights into understanding the functional mechanisms of the chicken-isolated *L. reuteri,* which also suggest its proteins as candidates for feed additives, food supplements, cosmetic and

Table 4 Selected immunogenic IL-2 and IL-10 inducing mutants of hypothetical proteins of chicken-isolated *Limosilactobacillus reuteri*

Protein source	Protein sequence	MP	pI	MW
QHPv.2.1	MNKESTVNSNSQSKIETIHASIDPKATEEEREEIIKRIENLKKQKQK	37	8.59	5482.89
	MNKESTVNSNSQSKIETIHASIDPKATEEEREEIIKWIENKKKQKQK	41	8.56	5527.93
QHPv.2.2	MDYQIRLETINDLPAIVDIFNQAIPLQVNGESAPIKVADRRE	8	4.39	4768.08
	MDYQIRLATINDLPAIVDHFNQAIPLQVNGESAPIKVADRRE	19	4.9	4734.02
QHPv.2.3	MGLETRRSEQLDKLFDQFAVDPKKPKATKKNDDKKEKDSKDKEKK	4	9.49	5323.72
	MGLVTRRSEQLDKLFDQFAVDPKKPKATKKNDDKFEKDSKDKEKK	35	9.54	5312.74
QHPv.2.4	MGQGQGAFKLSALQLKMSSAAVFAKLINDCDGLTERAMFS	16	8.51	4237.55
	MGQGQGAFKLSALQLNMSSAAVFAKLINDCDGLTERAMFS	16	6.44	4223.48
QHPv.2.5	MLISWFLGLLTCEIYLPLQLWAWLALIIGVGFFVWHIMLRCE	13	5.41	5010.9
	MLISWFLGLETCTIYLPLQLWAWLALIIGVGFFVWHIMLRCE	10	5.41	4998.84
QHPv.2.6	GTGLKAQIKKYLRSNGINMITNEQGRSIRLGNAKTQDLLKVAVKHGF	1	10.71	5169.8
	ETGLKAQIKKYLRSNGINMITNEQGRSIRLGNAKTQDLLKVAVKHGF	1	10.38	5241.86
QHPv.2.7	MIKTDKDTKTLEAQAVDLELRLLNIDLEH	18	4.65	3367.33
	MIKTDKDTKTLEAQAVDFELRLLNIDLEH	18	4.65	3401.34
QHPv.2.8	MPNWFAIFGMVFGFVILPLILFWLTRVERAENDERVK	27	6.6	4456.91
	MPNWFAIFGMVFKFVILPLILFWLTRRERAENDERVK	13	9.99	4585.08
QHPv.2.9	MAKYTVELSEEDIQMIKDCHSKNPSIMKCMNDAKKVED	26	5.09	4404.66
	MAKYTVELSEEDIQMIKDCHSKNPSCMKAMNDAKKVED	26	5.09	4362.57
QHPv.2.11	MNLINRIGEELGFAFNFVKVGLELACFLVAIADLFM	23	4.41	4020.4
	MNLINRIGEELGFAFNFVKVGLLLACFFVAIADLFM	28	4.68	4038.46
QHPv.2.12	MTVNDENGTLTAVFANSERIENKTLLNAFENTVNQRVEQYGQYITVL	36	4.43	5350.65
	MTVNDENGTLTAVFANSERIENKTLLNAFENTVNQNVEQYGQYITVL	36	4.15	5308.57
QHPv.2.14	MKFIAMLMVPCMFIGALLIKPTLIGVGLFVTSLIVEFLIIYLVTYMLEAH	11	7.06	5619.96
	MKFIAMLMVPVMFIGALEIKPTLIGVGLFVTSLIVEFLIIYLVTYMLEAH	18	5.51	5631.91
QHPv.2.16	MNKESTVNSNSQSKIETIHASIDPKATEEEREEIIKWIENKKKQKQK	41	8.56	5527.93
	MNKESTVNSNSQSKIETIHASIDPKATEEEGEEIIKWIENLKKQKQK	31	5.78	5413.79
QHPv.2.17	MDYQIRLETINDLPAIVDIFNQAIPLQVNGESAPIKVADRRE	8	4.39	4768.08
	MDYQIRLATINDLPAIVDHFNQAIPLQVNGESAPIKVADRRE	19	4.9	4734.02

MP Mutant position, *pI* Isoelectric point, *MW* Molecular weight

therapeutic purposes. However, the findings being generated through in silico analyses, necessitate the need for wet lab experimental studies to verify the results presented, for which the present study has laid a strong foundation.

Methods

Sequence retrieval

The assembled whole genome nucleotide sequence of the chicken-isolated *Limosilactobacillus reuteri* genome (accession CP059275 and version CP059275.1) was retrieved from NCBI. Bacteria isolation was from feces of reared broiler chicken in Nigeria and the genome was sequenced using Illumina MiSeq sequencing technology. Protein families were sorted in PATRIC. Heat and cold shock and hypothetical proteins were randomly selected for further analyses. The selected explored proteins are presented in Table 5.

Multiple sequence alignment

Multiple sequence alignment of the hypothetical proteins was done with Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo), which uses seeded guide trees and Hidden Markov Model profile-profile techniques to generate alignments and a relationship tree was generated for the hypothetical proteins. This was done for sequence similarity check and for understanding the relationships among the proteins.

Transmembrane topology and sub-cellular localization prediction

Transmembrane topology prediction was evaluated using DeepTMHMM, (https://dtu.biolib.com/DeepTMHMM/) a deep learning protein language model-based algorithm that detects and predicts the topology of both alpha-helical and beta barrels proteins over all domains of life [59]. Sub-cellular localization was evaluated using PSORTb v3.0 [60].

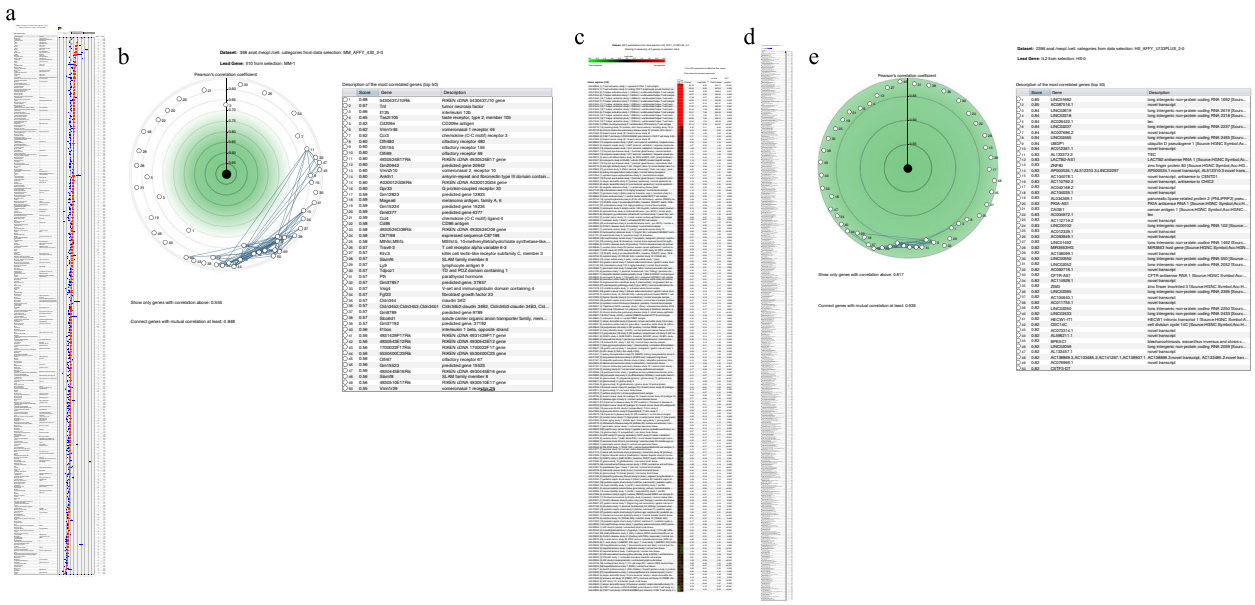


Fig. 5 IL-2 and IL-10 expression level and co-expression across various conditions: **a** scatter plot showing IL-2 and IL-10 expression (Log2 ratio) in *Mus musculus* according to anatomical parts and cell types; **b** a circular view of positively co-expressed genes with IL-10 in *Mus musculus* across various anatomical, neoplasm and cell categories; **c** a heatmap showing IL-2and IL-10 expression (Log2 ratio) in *Homo sapiens* across various perturbations; **d** a heatmap showing IL-2 and IL-10 expression level (linear scale) across various cancer categories in *Homo sapiens*; e. a circular view of positively co-expressed genes with IL-2 across anatomical parts, neoplasm and cell types in *Homo sapiens*

Table 5 Evaluated proteins of chicken-isolated *Limosilactobacillus reuteri* genome

Proteins	Designation
fig|1598.3022.peg.365| Cold shock protein of CSP family	CSP1
fig|1598.3022.peg.1253| Heat shock protein 10 kDa family chaperone GroES	HSP10kda
fig|1598.3022.peg.1252| Heat shock protein 60 kDa family chaperone GroEL	HSP60kda
fig|1598.3022.peg.1668| Heat shock protein GrpE	HSPGrpE
fig|1598.3022.peg.1900| Heat shock protein HtpX	HSPHtpX
fig|1598.3022.peg.703| Cold shock protein of CSP family	CSP2
fig|1598.3022.peg.1674| hypothetical protein	QHPv.2.1
fig|1598.3022.peg.1606| hypothetical protein	QHPv.2.2
fig|1598.3022.peg.1944| hypothetical protein	QHPv.2.3
fig|1598.3022.peg.2105| hypothetical protein	QHPv.24
fig|1598.3022.peg.2195| hypothetical protein	QHPv.2.5
fig|1598.3022.peg.218| hypothetical protein	QHPv.2.6
fig|1598.3022.peg.2256| hypothetical protein	QHPv.2.7
fig|1598.3022.peg.2313| hypothetical protein	QHPv.2.8
fig|1598.3022.peg.2318| hypothetical protein	QHPv.2.9
fig|1598.3022.peg.284| hypothetical protein	QHPv.2.11
fig|1598.3022.peg.27| hypothetical protein	QHPv.2.12
fig|1598.3022.peg.357| hypothetical protein	QHPv.2.13
fig|1598.3022.peg.34| hypothetical protein	QHPv.2.14
fig|1598.3022.peg.37| hypothetical protein	QHPv.2.15
fig|1598.3022.peg.1674| hypothetical protein	QHPv.2.16
fig|1598.3022.peg.1606| hypothetical protein	QHPv.2.17

Physicochemical properties and three-dimensional structures

Physicochemical properties of the evaluated proteins were analyzed with ExPASy ProtParam, www.web.expasy.org/protparam [14] and the three-dimensional tertiary structures of the evaluated proteins were obtained from Alphafold 2.0, https://alphafold.ebi.ac.uk/ [61]. Physical properties are used to characterized proteins in order to understand them.

IL-2 and IL-10 -inducing potential

Each of the selected hypothetical proteins of chicken-isolated *Limosilactobacillus reuteri* was evaluated for IL-2 and IL-10-inducing capability using IL2pred (https://webs.iiitd.edu.in/raghava/il2pred/predict.php) and IL10pred (https://webs.iiitd.edu.in/raghava/il10pred/predict3.php) respectively. Four hundred mutants were generated from each selected hypothetical protein of chicken-isolated *Limosilactobacillus reuteri* with a single site amino acid mutation per mutant. They were evaluated for IL-2 and IL-10-inducing potential and the top fifty according to their ML and SVM values. They were further analyzed for immunogenicity analysis. Of all the 50 evaluated, the top 10 immunogenic and IL-2 and IL-10-inducing mutants from each group were considered for statistical analysis.

Gene expression

To further understand conditions influencing the expression of IL-2 and IL-10, gene expression and co-expression analyses for *Homo sapiens* (human) and *Mus musculus* (mouse) under various conditions were performed using GENEVESTIGATOR, using HS_AFFY_U133PLUS_2-0 and MM_AFFY_430_2-1 platforms for *Homo sapiens* and *Mus musculus* respectively.

Statistical analysis

The top 10 selected mutants of selected hypothetical proteins of the chicken-isolated *Limosilactobacillus reuteri* were organized in a completely randomized design and subjected to a one-way analysis of variance of the SAS general linear model [1]. Significant means were analyzed using Duncan's Multiple Range Test. Statistical significance was set at $p < 0.05$ [62].

Abbreviations

AA	Amino acid
APCs	Antigen-presenting cell
CSP	Cold shock protein
EC	Extinction coefficient
HSP	Heat shock protein
IBD	Inflammatory bowel disease
II	Instability index
AI	Aliphatic index
IL-2	Interleukin-2
IL-10	Interleukin-10
LAB	Lactic acid bacteria
MP	Mutant position
MW	Molecular weight
pI	Isoelectric point
SLE	Systemic lupus erythematosus
TH1	T helper 1
TH17	T helper 17
TH2	T helper 2
TR1	T regulatory type 1 cells
Treg	Regulatory T cells

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Authors' contributions

Conceptualization, analysis, writing, review and editing: IOA.

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Availability of data and materials

The datasets used in this manuscript are included.

Declarations

Competing interests

The authors declare no competing interests.

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