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RESEARCH ARTICLE OPEN ACCESS

Drug Target Identification for *Listeria monocytogenes* by Subtractive Genomics Approach

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Abstract

We discover essential enzymes catalyzing critical metabolic reactions as potential drug targets, which may help to fight *Listeria* infections and their associated secondary infections extensively and effectively. A comparative metabolic pathway approach has been applied to identify and determine putative drug targets against *Listeria monocytogenes*. For this, enzymes unique to pathogenic pathways of *L. monocytogenes* EGD-e were determined using the KEGG database. They were further refined by selecting enzymes with sequences non-homologous to the host *Homo sapiens* and analysing their essentiality to the pathogen's survival. We report 15 essential pathogen-host non-homologous proteins as putative drug targets that can be exploited for development of specific drug targets or vaccines against multidrug resistant strains of *L. monocytogenes.* Finally, four essential enzymes from the pathogen: UDP-N-acetylglucosamine 1-carboxyvinyltransferase, Acetate kinase, Phosphate acetyltransferase, and Aspartate kinase were reported as novel putative targets for vaccine and drug development against *L. monocytogenes* infections. Unravelling novel target proteins and their associated pathways by comparing metabolic pathway analysis between *L. monocytogenes* EGD-e and host *H. sapiens*, develops the novelty of the work towards broad spectrum putative drug targets. This research design yields putative drug target critical enzymes that turn out to be fatal to the pathogen without interacting with the host machinery.

Keywords: *Listeria monocytogenes* EGD-e*, Homo sapiens,* Comparative Metabolic Pathway Analysis, Critical Enzymes, Novel Putative Drug Targets

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INTRODUCTION

Humans are victim of a large number of microbial pathogens through infection in the respiratory tract, gastrointestinal tract, or genital tract epithelium. These pathogens further migrate to secondary locations through lymph and blood to cause diseases in the liver, spleen, bones, and central nervous system (CNS).[1] *Listeria monocytogenes* is a Gram-positive, rod-shaped, foodborne pathogenic bacterium that is associated with listeriosis, CNS infection, meningitis, sepsis, liver infection, spleen infection and premature birth/ abortions.[2,3] It is also associated with immune response based diseases like Rheumatoid Arthritis (RA) and Inflammatory Bowel Disease (IBD).[4-6] *L. monocytogenes* has been found proliferating in colon of a patient suffering from IBD, much higher than in a healthy individual. Resistant biofilms of *Listeria* have been reported on all forms of synthetic and biological surfaces, including gut epithelium.[7,8] *L. monocytogenes* enters the human host through the ingestion of contaminated food and reaches the intestine. It crosses the intestinal epithelial barrier and enters the bloodstream, from where it causes secondary infection in several organs such as liver, spleen, bones, brain etc.[2] In South Africa, 978 people were found positive for listeriosis infection which led to 674 hospitalizations and 183 deaths were recorded in 2018.[9] Listeriosis outbreaks due to food borne infections have been recorded regularly in Turkey.[10] 1,763 cases of listeriosis from 27 states were recorded by European Food Safety Authority (EFSA) in 2013, leading to 191 deaths.[11] The fatality rate of listeriosis is about 20-30% among various parts of the world in 2018-19.[12-14] The current treatment of such *Listeria* infections depends on the use of antibiotics, which turn out to be ineffective and have risks of developing drug-resistant strains. At first, the most common treatment for *Listeria* infections was administration of antibiotics such as penicillin G and/or ampicillin along with aminoglycoside such as gentamicin or kanamycin. Subsequently, a combination of trimethoprim and a sulphonamide was also used as a therapy.[3,15] Initially, the majority of the *L.monocytogenes* strains (isolated from various sources) were susceptible to antibiotics, active against the Gram positive bacteria. The first drug resistance was observed against the antibiotic tetracycline in 1988.[16,17] A multidrug resistant strain of *L. monocytogenes* was also isolated in France in 1988.[16,17] Consequently, several multidrug resistant strains of *Listeria* have been discovered from environment, food sources and human listeriosis gut/stool samples.[17,18] *L. monocytogenes* strains isolated from several food items (including ready-to-eat food) were reported to be resistant to several antibiotics such as ampicillin, clindamycin, nalidixic acid, penicillin (100%) and oxacillin (94.1%).[19] The indiscriminate use of effective antibiotics in humans and animals has resulted in an exposure to high concentrations of these antibiotics, leading to resistance development in pathogenic strains of *Listeria* through gene alterations and transposons.[20,21] The infection of these multidrug resistant strains of *L. monocytogenes* through various food sources may pose a serious threat to public health.[9] So, this research attempts to find enzymes catalyzing essential metabolic reactions as potential drug targets, which may help to fight *L. monocytogenes* infections and their associated secondary infections extensively and effectively.

The easy accessibility of human genome sequences, complete genome sequences of several human infecting pathogens, and availability of several computational tools for their *in silico* analysis helps us to identify biomarkers and potential drug targets to combat these pathogens. Among these, comparative metabolic pathway analysis interpretations involve the understanding of the organism's physiology, intracellular procedures, and networks, which can find the specific target molecules responsible for their survival, vital functions, or virulence. This can further be used to develop specific and effective drugs against the pathogens. Metabolic pathways illustrate how the bio-molecular units interact with each other to carry out the functions required for the survival, reproduction, and other organism-specific activities.[22] Understanding the phenotype and function of organisms requires a detailed analysis of the metabolic pathways involved along with the study of single genes, as the expression of all the protein metabolites is a result of the action of enzymes catalyzing their interconversion. Proteins are the functional biomolecular element of the cell which converts

the genetic information into practical reality. They are involved in gene regulation, cell metabolism, transport of nutrients, signal transduction, and enzymatic catalysis. Hence, the pathogen-specific proteins/enzymes can be used as broad-spectrum potential drug targets against these pathogens.

In the present study, comparative metabolic pathway analysis was implied between *L. monocytogenes* EGD-e (Reference genome) and *H. sapiens* with the aim to select enzymes unique to *L. monocytogenes* EGD-e, which are non-homologous to host. These enzymes can work as putative targets for the development of drugs to eradicate these pathogen without interacting with the host machinery. These enzymes were further analysed for their essential role in survival of the pathogen. As targeting the critical enzymes turn out to be fatal to the pathogen and help developing broad-spectrum drugs. Different comparative genomics and transcriptomics studies and comparative proteomic analysis have been performed between different strains of *L. monocytogenes* or between different species of *Listeria* with diverse objectives, but unravelling novel target proteins and their associated pathways by comparing metabolic pathway analysis between *L. monocytogenes* EGD-e and host *Homo sapiens*, develops the novelty of the work towards broad spectrum putative drug targets.

MATERIALS AND METHODS

Metabolic Pathway Analysis of Pathogen and Host

Metabolic pathways of *L. monocytogenes* EGD-e and *H. sapiens* were retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/).[23] A manual assessment was performed to classify common and unique metabolic pathways between host and pathogen. The metabolic pathways present in pathogen but missing in the host were categorized as unique pathways, whereas the pathways present in both the pathogen and host were categorized as common pathways. The enzymes from unique metabolic pathways, having a sanctioned Enzyme Commission number (EC number), were mapped, and their protein sequences were extracted from the National Centre for Biotechnology Information (NCBI) genome database.[24]

Identification of Pathogen-Host Non-homologous Proteins

BLASTP[25] analysis (Protein-protein Basic Local Alignment Search Tool) was performed for protein sequences of enzymes from unique metabolic pathways against the *H. sapiens* proteome. The protein sequences with an e value cut-off of 5e-3 were considered as homologous to the pathogen and were excluded from the study. The protein sequences without a hit under this criterion were considered to have no significant homolog in *H. sapiens* and selected for further analysis.[26]

Identification of Essential Pathogen-Host Non-homologous Proteins

BLASTP analysis was performed between the obtained non-homologous protein sequences and prokaryotic essential genes from database of essential genes (DEG; http://www.essentialgene.org/) to yield essential pathogen-host non-homologous proteins [27]. The criteria of essentiality are as follows: evalue < 1e-10, bit score ≥ 100 and percentage identity ≥ 30.[26]

Subcellular Localization of Proteins

The exact position of these proteins in the cell was predicted using CELLO version 2.5 (subCELlular LOcalization predictor version 2.5).[28] CELLO applies the amino acid composition and di-peptide composition based on physico-chemical parameters of amino acids to predict the subcellular position of the proteins.[28] The gram-positive bacterial proteins have the following localization sites: the cell membrane, the cytoplasm, the extracellular space and the cell wall.

Structural Classification of the Unique Identified Target Proteins

The predicted enzymes as drug targets were further analysed and classified for their three dimensional structure and presence of any best possible ligands in their binding pockets. The available structures of the predicted potential drug targets were retrieved and studied from the RCSB

Protein Data Bank.[29] The research design for this study has been presented in Figure.

RESULTS AND DISCUSSION

KEGG Pathway Analysis Interpretations

KEGG database analysis revealed a total of 107 pathways associated with *L. monocytogenes* EGD-e. Most of these pathways were biosynthesis and metabolic pathways such as glycolysis, TCA cycle, carbohydrate metabolism, fatty acid biosynthesis and degradation, amino acid biosynthesis and degradation, nucleotide metabolism, peptidoglycan biosynthesis, vitamin metabolism, central dogma, nitrogen and sulfur metabolism etc. Also, other pathways associated with bacterial functions and virulence such as secondary metabolites biosynthesis, microbial metabolism in different environments, degradation of aromatic compounds, resistance to antibiotics such as vancomycin and beta-Lactam, cationic antimicrobial peptide (CAMP) resistance, quorum sensing, chemotaxis, NOD-like receptor signaling pathway, two-component system, and bacterial invasion of epithelial cells were reported (https://www.genome.jp/kegg/).

H. sapiens have 337 reported pathways in the KEGG database. These pathways consist of the eukaryotic biosynthesis and metabolic pathways. They also have several pathways related to drug and xenobiotics resistance and metabolism, signalling pathways, apoptosis,

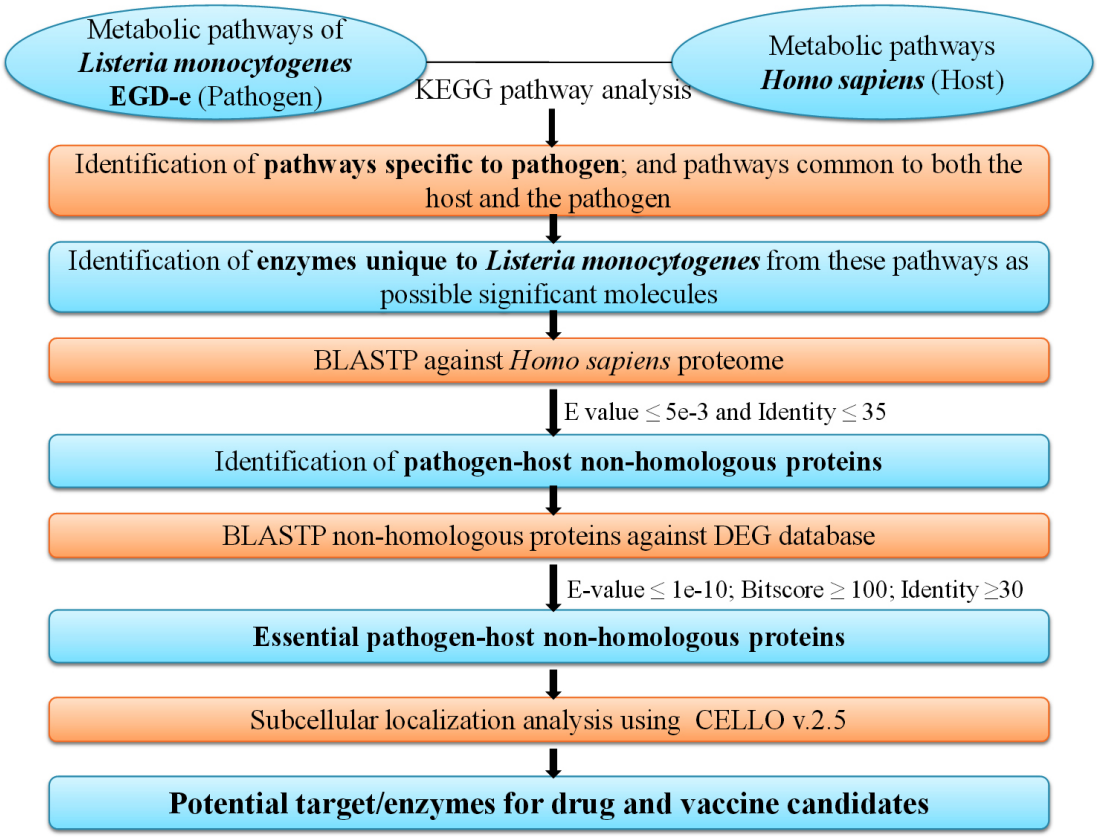

Figure. Schematic representation of subtractive genomics methodology for identification of potential drug and vaccine targets against *L. monocytogenes.* KEGG pathway analysis of metabolism for the pathogen (*L. monocytogenes*) and the host (*H. sapiens*) led to the subsequent identification of essential pathogen-host non-homologous enzymes/proteins which can be targeted as possible drug/vaccine candidates. (KEGG: Kyoto Encyclopedia of Genes and Genomes; BLASTP: Protein-protein Basic Local Alignment Search Tool; DEG: Database of Essential Genes; CELLO v2.5: subCELlular LOcalization predictor version 2.5)

Table 1. List of metabolic pathways common to both the pathogen *Listeria monocytogenes* EGD-e and host Homo sapiens (KEGG: Kyoto Encyclopedia of Genes and Genomes)

S. No.	Metabolic pathway	KEGG pathway ID
1	Glycolysis / Gluconeogenesis	lmo00010
2	Citrate cycle (TCA cycle)	lmo00020
3	Pentose phosphate pathway	lmo00030
4	Pentose and glucuronate interconversions	lmo00040
5	Fructose and mannose metabolism	lmo00051
6	Galactose metabolism	lmo00052
7	Starch and sucrose metabolism	lmo00500
8	Amino sugar and nucleotide sugar metabolism	lmo00520
9	Pyruvate metabolism	lmo00620
10	Glyoxylate and dicarboxylate metabolism	lmo00630
11	Propanoate metabolism	lmo00640
12	Butanoate metabolism	lmo00650
13	Inositol phosphate metabolism	lmo00562
14	Oxidative phosphorylation	lmo00190
15	Nitrogen metabolism	lmo00910
16	Sulfur metabolism	lmo00920
17	Fatty acid biosynthesis	lmo00061
18	Fatty acid degradation	lmo00071
19	Synthesis and degradation of ketone bodies	lmo00072
20	Glycerolipid metabolism	lmo00561
21	Glycerophospholipid metabolism	lmo00564
22	Arachidonic acid metabolism	lmo00590
23	Purine metabolism	lmo00230
24	Pyrimidine metabolism	lmo00240
25	Alanine, aspartate and glutamate metabolism	lmo00250
26	Glycine, serine and threonine metabolism	lmo00260
27	Cysteine and methionine metabolism	lmo00270
28	Valine, leucine and isoleucine degradation	lmo00280
29	Valine, leucine and isoleucine biosynthesis	lmo00290
30	Lysine degradation	lmo00310
31	Arginine biosynthesis	lmo00220
32	Arginine and proline metabolism	lmo00330
33	Histidine metabolism	lmo00340
34	Tyrosine metabolism	lmo00350
35	Phenylalanine metabolism	lmo00360
36	Tryptophan metabolism	lmo00380
37	Phenylalanine, tyrosine and tryptophan biosynthesis	lmo00400
38	beta-Alanine metabolism	lmo00410
39	Taurine and hypotaurine metabolism	lmo00430
40	Phosphonate and phosphinate metabolism	lmo00440
41	Selenocompound metabolism	lmo00450
42	D-Glutamine and D-glutamate metabolism	lmo00471
43	D-Arginine and D-ornithine metabolism	lmo00472
44	Glutathione metabolism	lmo00480
45	Other glycan degradation	lmo00511
46	Thiamine metabolism	lmo00730
47	Riboflavin metabolism	lmo00740
48	Vitamin B6 metabolism	lmo00750
49	Nicotinate and nicotinamide metabolism	lmo00760
50	Pantothenate and CoA biosynthesis	lmo00770

Table 1. Cont...

S. No.	Metabolic pathway	KEGG pathway ID
51	Biotin metabolism	lmo00780
52	Lipoic acid metabolism	lmo00785
53	Folate biosynthesis	lmo00790
54	One carbon pool by folate	lmo00670
55	Porphyrin and chlorophyll metabolism	lmo00860
56	Ubiquinone and other terpenoid-quinone biosynthesis	lmo00130
57	Terpenoid backbone biosynthesis	lmo00900
58	RNA polymerase	lmo03020
59	Ribosome	lmo03010
60	Aminoacyl-tRNA biosynthesis	lmo00970
61	Protein export	lmo03060
62	Sulfur relay system	lmo04122
63	RNA degradation	lmo03018
64	DNA replication	lmo03030
65	Base excision repair	lmo03410
66	Nucleotide excision repair	lmo03420
67	Mismatch repair	lmo03430
68	Homologous recombination	lmo03440
69	ABC transporters	lmo02010
70	NOD-like receptor signaling pathway	lmo04621
71	Bacterial invasion of epithelial cells	lmo05100

muscle contraction, platelet activation, circadian cycle, hormone secretion and immune responses. A number of pathways were specifically related to diseases and infections such as Alzheimer disease, Parkinson disease, Huntington disease, prion diseases, drug addiction, *Vibrio cholerae* infection, Pathogenic *Escherichia coli* infection, *Salmonella* infection, malaria, tuberculosis, pathways in cancer, asthma, inflammatory bowel disease, and rheumatoid arthritis etc (https://www.genome.jp/kegg/).

The comparative metabolic pathway analysis using the KEGG database resulted in 28 pathways that were unique to the pathogen *L. monocytogenes* EGD-e, whereas 71 pathways, common to both *L. monocytogenes* EGD-e and *H. sapiens* (Table 1 and Table 2 respectively). A total number of 180 enzymes with valid EC numbers were identified from the unique pathogen pathways (Supplementary Table 1).

Determination of Essential Pathogen-Host Non-homologous Proteins

The unique pathogen enzymes were then compared with host proteome to identify host non-homologous proteins. Out of 180 enzymes, 120 enzyme sequences revealed d" 35% identity with human proteome, and 52 enzyme sequences did not show any hit against the human proteome (Supplementary Table 2). Enzymes which are unique to *L. monocytogenes* and also do not show any or significant homology to the host proteome may act as effective drug targets as these drug/vaccine candidates have reduced risk of any unwanted interaction with the host proteins. Hence, these drugs will be safe and not adversely affect the human host metabolism.

Essential genes are the least number of genes which are obligatory for the existence of any organism.[30] Essential genes determined for 48 bacterial species have been listed in DEG. This essential genes list can be directly extracted and BLAST analysis can be performed. Alternatively, a common list of prokaryotic essential genes can be used for analysis of other organisms which are not listed in DEG, as performed in this study (DEG; http://www.essentialgene.org/). The knockout of any bacterial essential gene can produce lethal phenotypes, so essential genes may act as significant drug targets.[31] This can also be exploited for development of specific drug targets or vaccines against multidrug resistant strains such

Table 2. List of metabolic pathways which are unique to the pathogen *Listeria monocytogenes* EGD-e (KEGG: Kyoto Encyclopedia of Genes and Genomes)

S.No.	Metabolic pathway	KEGG pathway ID	No. of proteins
1	C5-Branched dibasic acid metabolism	lmo00660	7
2	Methane metabolism	lmo00680	17
3	Secondary bile acid biosynthesis	lmo00121	2
4	Lysine biosynthesis	lmo00300	17
5	Cyanoamino acid metabolism	lmo00460	5
6	D-Alanine metabolism	lmo00473	5
7	Peptidoglycan biosynthesis	lmo00550	16
8	Polyketide sugar unit biosynthesis	lmo00523	4
9	Carbapenem biosynthesis	lmo00332	2
10	Monobactam biosynthesis	lmo00261	6
11	Streptomycin biosynthesis	lmo00521	7
12	Acarbose and validamycin biosynthesis	lmo00525	2
13	Novobiocin biosynthesis	lmo00401	3
14	Benzoate degradation	lmo00362	3
15	Aminobenzoate degradation	lmo00627	3
16	Chloroalkane and chloroalkene degradation	lmo00625	1
17	Xylene degradation	lmo00622	2
18	Styrene degradation	lmo00643	3
19	Naphthalene degradation	lmo00626	1
20	Phosphotransferase system (PTS)	lmo02060	58
21	Bacterial secretion system	lmo03070	3
22	Two-component system	lmo02020	26
23	Quorum sensing	lmo02024	12
24	Bacterial chemotaxis	lmo02030	2
25	Flagellar assembly	lmo02040	1
26	beta-Lactam resistance	lmo01501	3
27	Vancomycin resistance	lmo01502	6
28	Cationic antimicrobial peptide (CAMP) Resistance	lmo01503	6

as *L. monocytogenes.* Some essential genes may be conserved over a number of related species and are potential targets for development of broad spectrum antibiotics.[27,31] Hence, 172 host-non-homologous enzyme sequences were analyzed for essentiality of *L. monocytogenes* using DEG. 98 enzymes were found to be essential enzymes of *L. monocytogenes* with an average identity of 49% to essential protein sequences of prokaryotes (Supplementary Table 3).

Subcellular Localization of Target Enzymes/ Proteins

Subcellular localization of these enzymes was determined by CELLO v.2.5, which may provide important information about the function of protein. The bacterial proteins/enzymes present in Gram-positive bacterial dataset are mostly localized in the cytoplasm and the cell membrane. Rest of the proteins are localized in the extracellular space and very few are found at the cell wall.[32] CELLO categorized our essential host non-homologous enzymes of *L. monocytogenes*, as presented in Supplementary Table 4.

Prediction of Potential Targets/ Enzymes for Drug and Vaccine Development

Out of the 52 non-homologous enzymes (with no identity match with *H. sapiens* proteome), 15 were recorded as essential enzymes for *L. monocytogenes* by DEG analysis with an average E value ≤ 1.4 e^{-23} and identity ≥ 47%. Further, we propose four *L. monocytogenes* enzymes as putative drug targets, completely non-homologous to human and critically important to survival of pathogen (with identity ≥ 60% to the essential

Table 3. Potential drug target enzymes from *Listeria monocytogenes*EGD-e, showing their essential role in survival of the pathogen and non-homologous nature to host Homo sapiens. KEGG: Kyoto Encyclopedia of Genes and Genomes)

S.No.	*Listeria monocytogenes* EGD-e enzyme name	KEGG ID	Non homologous (Yes/No) and Percentage Identity (%) to Homo sapiens	Essential (Yes/No) and Percentage Identity (%) to *Listeria monocytogen* esEGD-e	Sub-cellular localization
1	K00790 UDP-N-acetylglucosamine 1-carboxyvinyltransfera se [EC:2.5.1.7] |	lmo:lmo2526	Yes 0%	Yes 72.55%	Cytoplasmic
2	K00925 acetate kinase [EC:2.7.2.1] |	lmo:lmo1581	Yes 0%	Yes 71.61%	Cytoplasmic
3	K00625 phosphate acetyltransferase [EC:2.3.1.8] |	lmo:lmo2103	Yes 0%	Yes 63.11%	Cytoplasmic
4	K00928 aspartate kinase [EC:2.7.2.4] |	lmo:lmo1235	Yes 0%	Yes 61%	Cytoplasmic
5	K02760 PTS system, cellobiose-specific IIB component [EC:2.7.1.196 2.7.1.205] |	lmo:lmo2373	Yes 0%	Yes 57.73%	Cytoplasmic
6	K02769 PTS system, fructose-specific IIB component [EC:2.7.1.202] |	lmo:lmo0427	Yes 0%	Yes 57.29%	Cytoplasmic
7	K04041 fructose-1,6-bisphosphatase III [EC:3.1.3.11] |	lmo:lmo0830	Yes 0%	Yes 51.85%	Cytoplasmic
8	K02799 PTS system, mannitol-specific IIB component [EC:2.7.1.197]	lmo:lmo2799	Yes 0%	Yes 50.11%	Membrane
9	K02777 PTS system, sugar-specific IIA component [EC:2.7.1.-] |	lmo:lmo1017	Yes 0%	Yes 48.41%	Cytoplasmic
10	K00887 undecaprenol kinase [EC:2.7.1.66] |	lmo:lmo1464	Yes 0%	Yes 46.4%	Membrane
11	K02755 PTS system, beta-glucoside-specific IIA component [EC:2.7.1.-]	lmo:lmo2772	Yes 0%	Yes 42.11%	Membrane
12	K00425 cytochrome bd ubiquinol oxidase subunit I [EC:7.1.1.7]|	lmo:lmo2718	Yes 0%	Yes 41.45%	Membrane
13	K01624 fructose-bisphosphate aldolase, class II [EC:4.1.2.13] |	lmo:lmo0359	Yes 0%	Yes 39.1%	Cytoplasmic
14	K00003 homoserine dehydrogenase [EC:1.1.1.3] |	lmo:lmo2547	Yes 0%	Yes 38.26%	Cytoplasmic
15	K12555 penicillin-binding protein 2A [EC:2.4.1.129 3.4.16.4] |	lmo:lmo2229	Yes 0%	Yes 33.22%	Membrane

prokaryotic sequences), as listed in Table 3. The nature and site of action of all these four enzymes was determined to be cytoplasmic. These enzymes tend to play a central role in the pathogenesis of *L. monocytogenes,* causing infections in humans.

UDP-N-acetylglucosamine 1-carboxyvinyltransferase is an enzyme of class transferases that catalyzes the transfer of enolpyruvate group to UDP-*N*-acetyl-α-D-glucosamine which is a significant and committing reaction of cell wall formation in bacteria.[33] It belongs to 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase family and subfamily of MurA. This enzyme also substantially involved in other biological processes like cell division, cell wall cycle, cell wall organization, and cell shape regulation along with peptidoglycan biosynthetic pathway.[33,34] (https://www.uniprot.org/).

Acetate kinase is involved in the acetyl-CoA biosynthesis pathway by catalyzing a sub-pathway reaction of phosphorylating acetate utilizing ATP and a divalent cation such as Mg^{2+} or Mn^{2+}. The reaction is summarized as: acetate + ATP = acetyl phosphate + ADP. The reverse reaction can also be catalyzed by this same enzyme. It is also involved in some metabolic intermediate biosynthesis, such as the organic acid metabolic process (https://www.genome.jp/kegg/). It has also been reported that acetyl phosphate levels in *L. monocytogenes* are directly involved in monitoring cell motility, chemotaxis, and resistant biofilm formation. In a study, Gueriri and co-workers developed acetate kinase mutants of *L. monocytogenes* with a blocked synthesis of acetyl phosphate. These mutants were reported with a phenotype of decreased ability of biofilm formation and diminished expression of flagellar protein biosynthesis and motility genes.[35] Also, other studies have reported that some *L. monocytogenes* virulence factors such as VirR/VirS can be activated by the production of acetyl phosphate in the cells.[36] The activity of acetate kinase has also been recorded in other pathogenic intestinal bacterial strains such as *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9. These pathogen bacteria have been found to be involved in causing IBD in the human host.[37]

The third drug target enzyme, phosphate acetyltransferase, shows transferase activity. Phosphate acetyltransferase, along with the subsequent action of acetate kinase, produces acetate from acetyl-CoA (or acetyl phosphate) and generates ATP.[38]

The Aspartate kinase enzyme performs kinase activity, transferase activity, and binding of ATP by the reaction: ATP + L-aspartate = 4-phospho-L-aspartate + ADP. Aspartate kinase is involved in cellular amino acid biosynthetic pathways such as lysine biosynthesis via diaminopimelate (DAP) formation, homoserine biosynthesis, and threonine biosynthetic pathway [39] (https://www.uniprot.org/). These targets have not been used for drug/vaccine development to our best knowledge.

Other than these four novel drug targets, most of the other 11 enzymes have also been established as potential candidates for drug targets and have been reported in several other studies. Fructose-bisphosphate aldolase (FBA) class II, is a cytoplasmic or surface exposed bacterial enzyme catalysing the cleavage of fructose-1,6-bisphosphate to D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, an important reversible step in glycolysis and gluconeogenesis.[40] FBA is known to perform two or more unrelated functions in several bacterial species and hence is a moonlighting protein. FBA can play a significant role in binding to the host's cells and to host's proteins, subsequent generation of an immune response etc, and hence is involved in physiology and pathogenesis of the bacteria.[40] The structure and sequence of FBA remains conserved among same and different bacterial species, so it can be exploited to develop broad spectrum antibiotics/vaccines against a wide group of pathogenic bacteria.[41] Mendonca and coworkers reported FBA class II as a novel immunogenic surface protein and monoclonal antibody (mAb-3F8) against this protein for detection of the *Listeria* spp. and to distinguish *Listeria* from other pathogenic bacteria.[42]

The primary treatment for *L. monocytogenes* infected population is administration of β-lactam antibiotics (such as penicillins) which target a set of enzymes, the penicillin-binding proteins (PBPs) involved in peptidoglycan linking.[43] Our findings also report penicillin-binding protein 2A as a putative drug target. Peptidoglycan is a foremost element of the bacterial cell wall which is integral to the

cell structure and morphology. Peptidoglycan biosynthesis involves the creation of mesh like structure, which is facilitated by two steps: transglycosylation (elongation of glycan chain) and transpeptidation (peptide cross-linking the flanking glycan chains).[44] High molecular weight Class A PBP catalyze both of these reactions through their N-terminal glycosyltransferase and C-terminal transpeptidase domain.[44] Another research which applied several *in silico* approaches such as subtractive genomics and protein–protein interaction network topology, reported PDB4 along with 10 other proteins as a putative drug target in *L. monocytogenes* EGD-e proteome.[45]

We have also reported several phosphotransferase system (PTS) such as cellobiose-specific IIB component, mannitol-specific IIB component, fructose-specific IIB component, sugar-specific IIA component, and beta-glucoside-specific IIA component as putative drug targets. The PTS system is composed of a few soluble proteins and one membrane spanning protein, which are involved in the uptake/ transport of PTS carbohydrates by the cell.[46] It has also been reported to be actively involved in several regulatory functions such as catabolite repression, potassium transport, nitrogen and phosphate metabolism, antibiotic resistance, endotoxin production, biofilm formation, and virulence of several pathogens including *L. monocytogenes.*[46] PTS mediated sugar transport of cellobiose, mannose, and glucose has been reported to regulate PrfA activity, which is in turn the major transcription factor regulating the virulence gene in *L. monocytogenes.*[47] In a study based on comparative genomics of *Vibrio cholera,* several constituents of the PTS were described as drug and vaccine targets against the pathogen.[48] Similarly, six components of the PTS system were reported as putative drug targets in *Klebsiella pneumoniae* MGH78578 using the *in silico* approach.[49] Hence, the PTS system may act as an effective drug target for *L. monocytogenes* too.

Structural Classification of the Unique Identified Target Proteins

The available structures of the predicted potential drug targets were retrieved and studied from the RCSB Protein Data Bank.[29] The structures of the predicted drug targets were available on PDB either in *L. monocytogenes* or in other microbes. The minimum criteria for considering the three dimensional structure was on the basis of Identity >= 70%, Query coverage of the sequence as 80% and E Value <= 0.00. The crystal structure of PBP 4 from *L. monocytogenes* in the Ampicillin bound form (PDB ID: 3ZG8)[50] and PBP D2 from *L. monocytogenes* in apo form(PDB ID: 5ZQA)[51] were accessible in PDB. Both these structures were studied in expression system of *Escherichia coli*. Several ligands such as (2r,4s)-2-[(1r)-1-{[(2r)-2-Amino-2-Phenylacetyl]amino}-2-Oxoethyl]-5,5-Dimethyl-1,3-Thiazolidine-4-Carboxylic acid, glycerol and Di(hydroxyethyl) ether have been reported to bind to the A and B chains of this enzyme. Structures for other drug target proteins were available for different other organisms. For instance, Apo structure of fructose 1,6-bisphosphate aldolase from *Bacillus anthracis* str. 'Ames Ancestor' (PDB ID: 3Q94) can be studied at PDB.[52] This crystal structure was determined through X-ray diffraction experiment and the enzyme was found to be composed of A and B chains with two reported ligands (1,3-Dihydroxyacetonephosphate and acetate ion) interacting with the A chain of the protein. Similarly, the structure of Cytochrome BD-I ubiquinol oxidase from *Escherichia coli* (PDB ID: 6RX4) [53] and Homoserine Dehydrogenase from *Saccharomyces cerevisiae* (PDB ID: 1EBU) [54] were available. Two ligands (3-Aminomethyl-Pyridinium-Adenine-Dinucleotide and L-Homoserine) interacting with the D chain of the Homoserine Dehydrogenase enzyme and four ligands (Heme b/c, Cis-Heme d hydroxychlorin gamma-Spirolactone, 1,2-Dioleoyl-Sn-Glycero-3-Phosphoethanolamine, and Ubiquinone-8) binding with A and B chains of Cytochrome BD-I ubiquinol oxidase have been reported so far. Also, the structures of several enzymes reported as drug targets from the PTS system were retrieved for different organisms from PDB. Such as crystal structure of PTS System Cellobiose-specific Transporter Subunit IIB from *Bacillus anthracis* (PDB ID: 4MGE),[55] and structure of IIB domain of the mannitol-specific permease enzyme II from *Escherichia coli* (PDB ID: 1VKR)[56] can be studied from PDB. The crystal structure of the fructose specific IIB subunit of PTS system was available for *Bacillus subtilis* (PDB ID: 2R48).[57]

Similarly, the closest structures available for sugar-specific IIA component and beta-glucoside-specific IIA component of the PTS system were studied from PDB. The details of all these structures including the classification, expression system, mutations, gene names and ligand interactions have been compiled in Supplementary Table 5.

Out of the four novel drug targets predicted, the structures of two enzymes: UDP-N-acetylglucosamine 1-carboxyvinyltransferase and phosphate acetyltransferase were available for *L. monocytogenes.*[58,59] The crystal structure of UDP-N-acetylglucosamine 1-carboxyvinyltransferase (murA) from *L. monocytogenes* EGD-e (PDB ID: 3R38) was determined through X-ray diffraction experiments in the *Escherichia coli* BL21 expression system. Two ligands have been determined for this protein (Sulfate ion and Chloride ion) which bind to the chain A of the protein.[58] Similarly, the crystal structure of phosphate acetyl/butaryl transferase (from *L. monocytogenes* EGD-e) in complex with CoA (PDB ID: 3U9E) has been determined in *Escherichia coli* BL21(DE3) by X-ray diffraction studies. Four ligand molecules (Coenzyme A, Arginine, Glycerol, Chloride) have been known to interact with A and B chains of the protein[59] (Supplementary Table 5).

The structure for the third novel protein acetate kinase was found for the organism *Salmonella enterica* subsp. enterica serovar Typhimurium (PDB ID: 3SK3) in the RCSB Protein Data Bank. Citric acid and 1,2-Ethanediol ligands interact with the A and B chain of the enzyme.[60] Similarly, the structure for aspartate kinase was found for the organism *Pseudomonas aeruginosa* PAO1 (PDB ID: 5YEI) which was determined by X-ray diffraction in expression system *Escherichia coli* BL21(DE3). Three ligand molecules: Threonine, Lysine, and Glycerol are known to interact with the protein[61] (Supplementary Table 5). Unpinning the structure categorization and identifying the inhibitors for these target proteins opened different methods of research towards drug design and reverse vaccinology approach.

CONCLUSION

The comparative metabolic pathway analysis approach of *L. monocytogenes-H. sapiens* resulted in four novel putative target proteins: UDP-N-acetylglucosamine 1-carboxyvinyltransferase, acetate kinase, phosphate acetyltransferase, and aspartate kinase, which were very high in essentiality index with the pathogen and non-homologous nature with the host. Other 11 enzymes on the list are also significant putative drug targets and some of them have been reported by prior studies as well. The predicted potential drug target enzymes from *L. monocytogenes* will not interact with host machinery and also perform essential functions such as peptidoglycan biosynthesis, cell motility, chemotaxis, resistant biofilm formation, virulence, bacterial pathogenicity, amino acid biosynthesis, cell division, and cell wall organization. Therefore, drug development against these targets to combat *L. monocytogenes* infections will be very promising. Unravelling novel target proteins and their associated pathways by comparing metabolic pathway analysis between *Listeria monocytogenes* EGD-e and host *Homo sapiens*, develops the novelty of the work towards specific and broad spectrum putative drug targets. In addition to this, a detailed further analysis of these potent target proteins in terms of *in-vivo* and *in-vitro* approach will attain new and unique generation of biomolecules against the diseases caused by *L. monocytogenes*.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.17.3.55

Additional file: Additional Table S1- S5.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

NC and SS designed the experiments. NC

performed the experiments. NC analyzed the data and wrote the manuscript. NC and TQ revised the manuscript. All authors read and approved the final manuscript for publication.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or in the supplementary files.

ETHICS STATEMENT

Not applicable.

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