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# A COMBINATION OF IN SILICO SUBTRACTIVE AND REVERSE VACCINOLOGY APPROACHES REVEALS POTENTIAL VACCINE TARGETS IN *Corynebacterium pseudotuberculosis*

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**Abstract:** *Corynebacterium pseudotuberculosis* is responsible for caseous lymphadenitis (CLA) in ruminants, which contributes to significant economic loss to some countries. Although extensive efforts are in progress to develop vaccines against this pathogen, to date no effective vaccine has been developed. The present work identifies potential vaccine targets in *C. pseudotuberculosis*. A combination of *in silico* subtractive and reverse vaccinology approaches were employed to identify non-host homologous essential *C. pseudotuberculosis* proteins, which also fulfilled at least two of the following criteria: i) transmembrane domain, ii) signal peptide and iii) antigenic epitope. Results demonstrated that a total of 18 non-host homologous essential *C. pseudotuberculosis* proteins were considered as potential vaccine targets, including lipoprotein signal peptidase, ABC transporter ATP-binding protein, phosphate ABC transporter permease PstA, phosphoglucosamine mutase, inner membrane protein translocase component YidC and C4-dicarboxylate transporter DctA. Further analysis showed the potential role of inner membrane protein translocase component YidC as a hub protein. Homology modelling of this protein yielded a good model based on related structures from the Protein Data Bank. The development of a protective vaccine against *C. pseudotuberculosis* using non-host homologous essential proteins may offer an advantage to control caseous lymphadenitis in ruminants.

Keywords: Antigenicity, caseous lymphadenitis, *Corynebacterium pseudotuberculosis*, *in silico* subtractive, reverse vaccinology, vaccine.

## Introduction

*C. pseudotuberculosis* is a Gram-positive facultative pathogen that causes CLA in sheep and goats. It has been shown to form biofilm on various surfaces, such as glass, plastic and silicon (Yaacob *et al.,* 2021). Inflamed lymph nodes and abscess formation are commonly observed in CLA infections. The disease is transmitted via different routes such as intradermal, subcutaneous, oral and nasal routes (Jesse *et al.*, 2016). Many reports have shown that gradual weight loss, reduction of fertility, delayed development, and occasional death of animals resulting from CLA infections contribute to significant economic loss to some countries (Cetinkaya *et al*., 2002; Zavoshti *et al.,* 2012). Over the past few decades, clinical detection of *C. pseudotuberculosis* in CLA - infected hosts often involves either serodiagnostic test, enzyme-linked-immunosorbent assay (ELISA) or polymerase chain reaction (PCR). Considering their critical roles in other microorganisms, essential proteins in *C. pseudotuberculosis* may have potential as vaccine targets.

Essential proteins are indispensable to support cellular life by maintaining central metabolism, DNA replication, protein synthesis, basic cellular structure and cellular transport. Their essentiality is known to be dependent on the environment in which an organism lives (Rancati *et al.*, 2017) and also type of organisms (Sharma *et al.,* 2014). There are several experimental approaches to study essential genes and proteins, such as directed deletion of genes

and random mutagenesis using transposons. However, computational approaches, such as metabolic modelling, machine learning, comparative genomics and protein interaction network have gained greater attention due to their cost and time effectiveness.

Database of essential genes (DEG) contains more than 10,000 essential genes from 19 bacterial species (Zhang *et al.,* 2004). This bioinformatics tool relies on the use of basic local alignment search tool (BLAST) to identify homologous genes. Queried genes are considered as essential when their homologous genes are found in this database. Folador and colleagues (2016) studied essential proteins in *C. pseudotuberculosis* using protein interaction network and identified 181 essential proteins with a very high node-degree in the network. Identification of vaccine targets in *C. pseudotuberculosis* using the reverse vaccinology approach has previously been reported (Soares *et al.,* 2013; Araujo *et al.,* 2019). However, there is still no published work on identification of *C. pseudotuberculosis* essential proteins as vaccine targets using the large set of essential proteins from the DEG database searched against *C. pseudotuberculosis* proteome. Thus, the present study was carried out to identify non-host homologous essential proteins in *C. pseudotuberculosis* that meet the criteria for potential vaccine targets.

## Materials and Methods

### *Preparation of Protein Dataset*

*Acinetobacter baumannii* ATCC 17978, *Burkholderia thailandensis* E264, *Pseudomonas aeruginosa* PAO1 and *Campylobacter jejuni* ATCC 700819 were randomly selected for protein sequence preparation. Their essential proteins were retrieved in FASTA format from the DEG database.

### *Identification of Essential Proteins*

The essential protein dataset was subjected to BLASTp search (E-value < 1e-06; sequence identity > 30%) against *C. pseudotuberculosis* in the National Center for Biotechnology Information (NCBI) database. Redundant protein sequences from this analysis were manually removed.

### *Identification of Non-host Homologous Proteins*

Identified essential proteins of *C. pseudotuberculosis* were used in BLASTp search against *Homo sapiens* and *Ovis aries* in the NCBI database to identify essential proteins that are non-host homologous to host proteomes (E-value > 1e-06; sequence identity < 30%).

### *Analysis of Potential Vaccine Targets*

Non-host homologous essential proteins from *C. pseudotuberculosis* were further analyzed for their i) transmembrane domain, ii) signal peptide and iii) antigenicity using Transmembrane Hidden Markov Model (TMHMM) 2.0 (Krogh *et al.,* 2001), Phobius (Kall *et al.,* 2007) and VaxiJen 2.0 (Doytchinova & Flower, 2007) respectively. Identified proteins that fulfilled at least two of the criteria above were considered as ideal vaccine targets.

### *Functional Characterization*

The selected candidate was further analyzed for: i) protein interaction network using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (max interactions: 100; confidence level: high - 0.7) (Szklarczyk *et al.,* 2015), ii) secondary structure using Psipred (McGuffin *et al.,* 2000), iii) 3D modelling using SWISS-MODEL (Guex & Peitsch, 1997) and iv) model evaluation using PROCHECK Ramachandran plot (Guex & Peitsch, 1997).

## Results and Discussion

### *Essential Proteins*

Table 1 summarizes *in silico* analysis of *C. pseudotuberculosis* proteome. A total of 845 essential protein sequences were used as queries in BLASTp search against *C. pseudotuberculosis* proteome. This resulted in identification of 348 (41.2%) essential protein sequences of *C. pseudotuberculosis*.

### *Non-host Homologous Proteins*

The essential protein dataset was then used in homology search against human and sheep proteomes leading to the identification of 232 (27.5%) and 206 (25.6%) non-host homologous essential proteins respectively (Table 1). Those were absent in both human and sheep proteomes (85 protein sequences, 10.05%) were further considered in the analysis of transmembrane domain, signal peptide domain and antigenicity.

### *Potential Vaccine Targets*

A total of 18 (2.13%) non-host homologous essential *C. pseudotuberculosis* proteins were predicted to meet at least two of the following criteria: i) containing transmembrane domain; ii) containing signal peptide domain, and iii) containing antigenic epitope (Table 1). Lipoprotein signal peptidase, ABC transporter ATP-binding protein, phosphate ABC transporter permease PstA, phosphoglucosamine mutase, inner membrane protein translocase component YidC and C4-dicarboxylate transporter DctA were among the attractive vaccine targets (Table 2) and majority of them were found to associate with common metabolic pathways. Figure 1 shows representative topography of potential vaccine targets. Lipoprotein signal peptidase was predicted to contain both transmembrane and signal peptide domains, and phosphate ABC transporter permease PstA was predicted to contain only transmembrane domain.

### *Protein Interactions*

Inner membrane protein translocase component YidC was further analysed because it showed the highest antigenicity value, 0.71. Figure 2 displays protein interactions between inner membrane protein translocase component YidC and other proteins. This enzyme was predicted to form the protein interaction network with other 15 proteins. The network was mediated by 64 functional interactions and found to represent various biological pathways, such as bacterial secretion system, protein export and Quorum sensing. When K-means clustering algorithm (number of clusters: 7) was applied, inner membrane protein translocase component YidC was found to form a cluster with protein-export membrane protein SecG (secG), protein translocase subunit SecE (secE), protein translocase subunit SecY (secY), signal recognition particle protein (ffh), signal recognition particle receptor FtsY (ftsY), protein translocase subunit SecA (AIG10411.1) and protein translocase subunit SecA (AIG11844.1).

Table 1: A summary of *in silico* analysis of *Corynebacterium pseudotuberculosis* proteins

| Description | Number | % |
|---|---|---|
| Essential proteins from various pathogenic microorganisms | 845 | 100 |
| Essential proteins in *C. pseudotuberculosis* | 348 | 41.2 |
| Essential proteins without matches in human proteome | 232 | 27.5 |
| Essential proteins without matches in sheep proteome | 206 | 25.6 |
| Essential proteins that fulfilled at least two of the criteria for vaccine targets (transmembrane domain, signal peptide domain and antigenic epitope) | 18 | 2.13 |
| Essential proteins identified as potential vaccine targets | 18 | 2.13 |

Table 2: A list of non-host homologous essential *C. pseudotuberculosis* proteins that are considered as potential vaccine targets. Proteins that show score value of antigenicity > 0.4 are considered potential vaccine targets

| Accessions | Protein Names | Pathways | Antigenicity |
|---|---|---|---|
| A0A5C2NYB6 | Phosphoglucosamine mutase | Glucosamine | 0.6 |
| A0A4U9QQL2 | Twin-arginine translocase sub-unit TatC | Ion transport | 0.65 |
| A0A4U9QQQ3 | Magnesium and cobalt transport protein CorA transmembrane | Ion transport | 0.45 |
| A0A5C2NMA8 | Trk system potassium uptake protein | Ion transport | 0.48 |
| D9QB94 | Phosphoribosyl-AMP Cyclohydrolase | Histidine | 0.59 |
| D9QA12 | 3-isopropylmalate dehydratase large subunit | Leucine | 0.59 |
| D9QDT3 | 2-isopropylmalate synthase | Leucine | 0.51 |
| D9QBA2 | Histidinol-phosphate aminotransferase | Histidine | 0.41 |
| D9QAN9 | Shikimate kinase | Chorismate | 0.46 |
| D9QBY5 | Para-aminobenzoate synthase component I | Glutamine | 0.44 |
| D9QBX0 | Glutamate 5-kinase | Proline | 0.53 |
| D9QA22 | ATP-dependent DNA helicase | DNA replication | 0.43 |
| D9QAK2 | Phosphoenolpyruvate carboxylase | Oxaloacetate | 0.43 |
| A0A4U9QUK3 | ABC transporter ATP-binding protein | Ion transport | 0.48 |
| A0A4U9QE72 | Phosphate transport system permease protein PstA | Ion transport | 0.48 |
| A0A4U9QL42 | Aerobic C4-dicarboxylate transport protein | Ion transport | 0.48 |
| D9QDG4 | Inner membrane protein translocase component YidC | Protein transport | 0.71 |
| D9QB07 | DNA translocase ftsK | DNA replication | 0.57 |

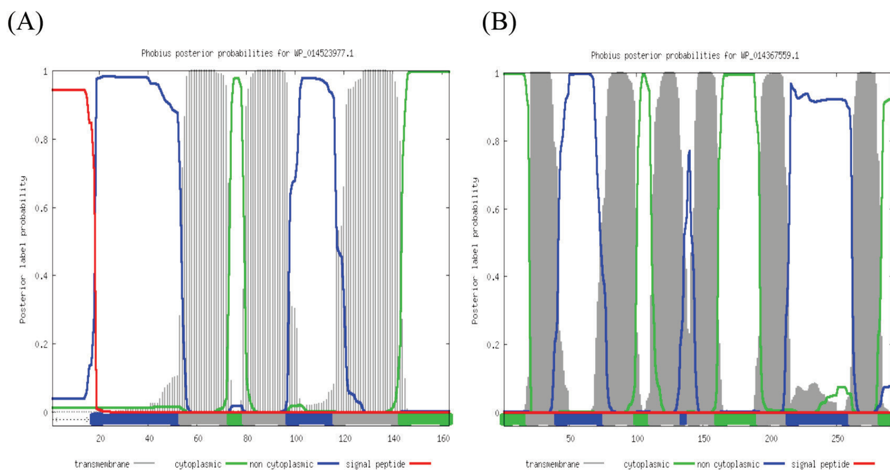
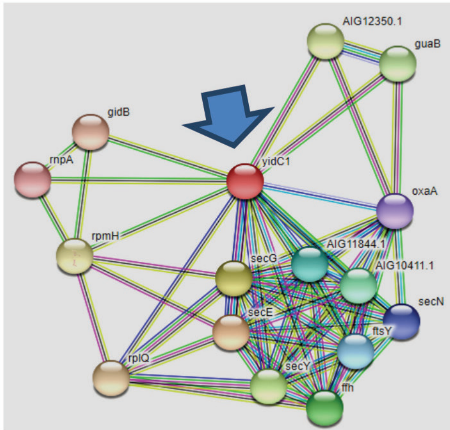



Figure 1: Representative topography of essential and non-host homologous proteins in *C. pseudotuberculosis.* A) Lipoprotein signal peptidase; B) phosphate ABC transporter permease PstA



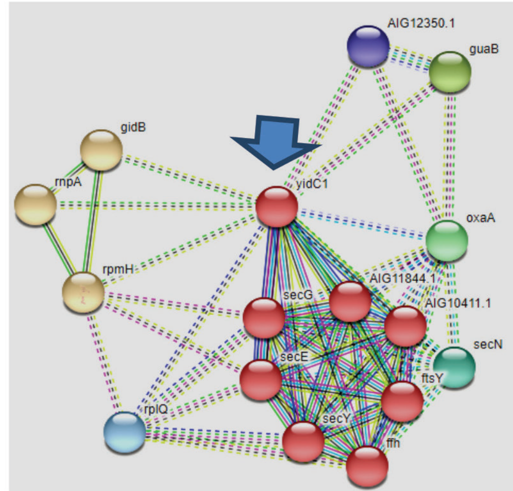
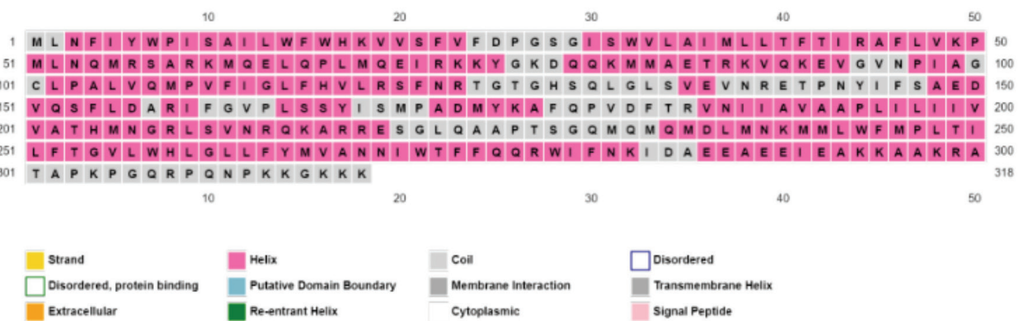




Figure 2: Functional interactions between inner membrane protein translocase component YidC and other proteins. Arrows indicate inner membrane protein translocase component YidC.
Left panel: A protein interaction network without K-means clustering; right panel: A protein interaction network with K-means clustering

***3D Model***

Figure 3 shows secondary structure and 3D model of inner membrane protein translocase component YidC while Table 3 shows a comparison of 3D models using different templates. Inner membrane protein translocase component YidC was predicted to be rich in helix structures whilst PDB_ID 3wo6.1.A was identified as the best template to construct a 3D model for inner membrane protein translocase component YidC. PROCHECK displayed 87.18% of residues in the most favoured regions. The 3D model using this template showed better quality than the other templates.

a) Secondary structure



b) Template-target alignment

c) 3D model

Figure 3: 3D modelling of inner membrane protein translocase component YidC. a) Secondary structure, b) target-template alignment and c) predicted 3D model (left) and Ramachandran plot (right)

Table 3: Different templates used to construct 3D model of inner membrane protein translocase component YidC. Asterisk (*) indicates the best template to construct a 3D model for inner membrane protein translocase component YidC

| | 3wo7.2.A | 3wo6.1.A* | 6al2.1.A |
|---|---|---|---|
| **Species** | *Bacillus halodurans* | *Bacillus halodurans* | *Escherichia coli* |
| **Sequence identity** | 26.36% | 26.94% | 26.88% |
| **Ramachandran favored regions** | 85.46% | 87.18% | 85.89% |
| **Ramachandran outliers** | 6.38% | 4.76% | 8.06% |

## Discussion

In the last few years, analysis of essential proteins in pathogenic microorganisms using subtractive proteomic approaches has received a great deal of attention. This approach is aimed at identifying potential drug or vaccine targets that are not present in the host. It has become the prime step towards discovery of effective therapeutic strategies which are free from adverse effects on the host. The present study compared known essential proteins from other microorganisms against the *C. pseudotuberculosis* proteome to identify essential proteins in this veterinary pathogen. The present study was carried out based on the fact that the proteins that are conserved in two or more bacterial species are almost certainly essential for cellular function (Mushigen & Koonin, 1996) . *In silico* subtractive approach taken in the present study has also been used in other computational works (Yahya *et al.,* 2014; Solanki & Tiwari, 2018; Othman & Yahya, 2019). *Homo sapiens* (human) and *Ovis aries* (sheep) proteomes were used in the *in silico* subtractive analysis because

*C. pseudotuberculosis* often transmits to these hosts in clinical settings (Folador *et al.,* 2016).

It has been established that survival of pathogens is greatly dependent on essential proteins, which play important roles intracellularly and extracellularly. The vast majority of the essential proteins perform a diverse range of functions in central metabolic pathways, cellular transport and cell-surface interactions. The present study demonstrated that a total of 438 *C. pseudotuberculosis* proteins were predicted to be essential and non-host homologous. Essential proteins are lethal for the organism when subject to a knock-out (Lu *et al.,* 2015), therefore, the identification of non-host homologous essential proteins herein represent an attractive dataset that could be exploited for drug development and vaccine production against *C. pseudotuberculosis* infection.

Membrane localization and secretion of pathogenic proteins are considered important factors for potential vaccine targets because membrane and secreted proteins are the first to be in contact with the host and trigger an immune response. In general, hosts respond to microbial infections by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB), initiating expression of genes involved in the defense mechanism and producing cytokines. Thus, prediction of proteins that are localized in bacterial outer membrane and secreted into extracellular matrix has become a valuable approach in reverse vaccinology. In the present study, a total of 18 non-host homologous essential proteins from *C. pseudotuberculosis* were identified as potential vaccine targets because they were predicted to contain transmembrane domain, signal peptide domain or antigenic epitope. These three criteria are important characteristics of vaccine targets (Leow *et al*., 2020). This finding is expected to provide a more reliable output compared to screening of the whole data set without considering prioritizing parameters (Barh *et al.,* 2011).

Lipoprotein signal peptidase is an enzyme involved in lipoprotein biosynthesis, which consists of three steps; (i) transfer of a diacylglyceride to the cysteine sulphydryl group of the unmodified prolipoprotein, (ii) cleavage of the signal peptide by signal peptidase II that forms an apolipoprotein and (iii) acylation of the α-amino group of the N-terminal cysteine of the apolipoprotein. This enzyme plays a role in lipoprotein posttranslational processing by cleaving signal peptides from bacterial membrane pro lipoproteins. In concordance with a few studies on signal peptidase, the identification of lipoprotein signal peptidase in *C. pseudotuberculosis* also showed the evidence of an essential and attractive vaccine target (Table 2). Vogeley and colleagues (2016) stated that this enzyme is essential in many pathogenic bacteria but has no equivalent in humans, making it an ideal drug target. Meanwhile, Moitinho-Silva and colleagues (2012) demonstrated that the signal peptidase in *Mycoplasma hyopneumoniae* is strongly immunogenic for mice and its antigenicity is proven.

Phosphoglucosamine mutase is an enzyme that catalyzes the formation of glucosamine-1-phosphate from glucosamine-6-phosphate that takes place in UDP-N-acetylglucosamine biosynthesis. In bacteria, N-acetylglucosamine is cross-linked to N-acetyl muramic acid forming a peptidoglycan layer. Activation of phosphoglucosamine mutase involves phosphorylation and glucosamine-1, 6-diphosphate as an intermediate. In the present study, phosphoglucosamine mutase in *C. pseudotuberculosis* was predicted to be essential, non-host homologous and antigenic (Table 2). This result corroborates Mengin-Lecreulx and Heijenoort (1996) findings that this enzyme is essential for peptidoglycan biosynthesis in *E.coli.* On the other hand, immunogenicity of phosphoglucosamine mutase in *Bordetella pertussis* has previously been reported by West *et al.* (2012).

ABC transporters are involved in export or import of a wide variety of substrates ranging from small ions to macromolecules. The major function of ABC transporters is to provide essential nutrients to bacteria. Structurally,

phosphate ABC transporter permease PstA in combination with permease PstC form a membrane channel that permits phosphate flow. On the other hand, ABC transporter ATP-binding protein functions in assisting ATP hydrolysis that generate energy for opening the membrane channel. In the present study, ABC transporter ATP-binding protein and phosphate ABC transporter permease PstA in *C. pseudotuberculosis* were predicted to be essential, non-host homologous and antigenic (Table 2). This result is in line with Braibant and colleagues (1996) showing essentiality of phosphate ABC transporter permease PstA in *E.coli.* This result also corroborates Pawelec and colleagues (1998) reporting that ABC transporter proteins are virulent and immunogenic, therefore they can be exploited as candidate subunits for vaccination against pathogenic bacteria.

In bacteria, C4-dicarboxylates, such as aspartate, malate, fumarate and succinate are utilized as carbon and energy sources under aerobic and anaerobic growth conditions. C4-dicarboxylate transporter DctA is a protein that functions in the transport of those dicarboxylates across bacterial membrane. Expression of this transport protein is dependent on growth phase and varies across bacterial species. In the present study, C4-dicarboxylate transporter DctA in *C. pseudotuberculosis* was identified as a potential vaccine target (Table 2). On the other hand, the immunogenicity of this transport protein was hampered due to scarce availability of data on this particular area. However, considering several evidences that many transport proteins spanning the bacterial membrane are potential vaccine candidates (Pawelec *et al,.* 1998; Caro-Gomez *et al.,* 2014; Leow *et al.,* 2020), it is possible that C4-dicarboxylate transporter DctA may also function as antigen and induce immune response in hosts.

Inner membrane protein translocase component YidC is an enzyme that functions in biogenesis of the membrane proteins in the cytoplasmic membrane of bacteria. It is known to mediate the transfer of transmembrane domains from the Sec-translocon into the lipid bilayer and assist the folding of inner membrane proteins. While the C-terminal region of transmembrane domain is essential for YidC activity, the large periplasmic domain is not essential for the function of YidC. The present study identified Inner membrane protein translocase component YidC in *C. pseudotuberculosis* as a potential vaccine target that were essential, non-host homologous and antigenic (Table 2). This result is consistent with Samuelson and colleagues (2000) that showed essentiality of YidC for *E. coli* viability and Caro-Gomez and colleagues (2014) identified inner membrane protein translocase component YidC as an antigen to encompass MHC class-I-binding peptides using *in silico* approach.

Numerous molecular processes are regulated via a large number of protein components modulated by protein-protein interactions, which refer to intentional direct and indirect contacts established between two or more proteins resulting in specific biochemical events. The STRING database is widely used in proteomic studies because it collects and integrates all information of protein-protein interactions, by consolidating known and predicted protein interaction data for a large number of organisms. In the present study, inner membrane protein translocase component YidC in *C. pseudotuberculosis* was predicted to have more than 10 interaction partners (Figure 2). Thus, this enzyme is considered as a hub protein in the protein interaction network (Yahya *et al.,* 2017; Othman & Yahya, 2019).

Homology modelling predicts the 3D structure of a query protein through the sequence alignment of template proteins. In general, the process of homology modelling involves four steps: target identification, sequence alignment, model building and model refinement. In the present study, the Ramachandran favoured region of inner membrane protein translocase component YidC model is considerably high (87.18%) (Figure 3). On the other hand, the high composition of helices in the inner membrane protein translocase component YidC structure may make the protein more flexible for folding

and increase protein interactions (Barh et *al.* 2011), corroborating our previous result showing the potential role of inner membrane protein translocase component YidC as a hub protein (Figure 2).

## Conclusion

The present study identified a total of 18 *C. pseudotuberculosis* proteins as potential vaccine candidates which were non-host homologous, essential and antigenic. Development of protective vaccine against *C. pseudotuberculosis* using these proteins may offer an advantage to control CLA in ruminants. The findings presented herein also serve as a basis for further research on pathogenesis of *C. pseudotuberculosis* while experimental validation for the identified proteins needs further attention.

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