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ORIGINAL RESEARCH

Assessment of chicken protection against *Campylobacter jejuni* infection by immunization with avirulent *Salmonella enterica* sv. Typhimurium strain producing *Campylobacter* CjaD/Pal protein

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Abstract: *Campylobacter jejuni* is a major food-borne pathogen, causing gastroenteritis worldwide. Chickens are considered to be one of the most common sources of human *C. jejuni* infection in developed countries. *Campylobacter* CjaD/Pal protein (annotated as Cj0113 in *C. jejuni* strain NCTC11168) is a highly immunogenic, membrane-located antigen, conserved among different strains, with the potential to provide broad protection against *C. jejuni* colonization. The present study examines the immunogenicity and the general efficacy of avirulent *S. enterica* sv. Typhimurium Δ*crp* Δ*cya* expressing *C. jejuni* CjaD as a chicken vaccine against *Campylobacter* colonization. The high copy number plasmid pYA3341 Asd$^+$ was used as a cloning vector. Here, 1- and 14-day old chickens were orally immunized with a delivery vector strain, expressing *C. jejuni* CjaD. Two weeks later, they were challenged with a wild-type *C. jejuni* strain isolated from chicken carcasses. This schedule of immunization induced significant levels of serum-specific IgG as well as mucosal intestinal sIgA as measured by ELISA tests using *Campylobacter* membrane proteins as a coating antigen. Nevertheless, protection experiments did not result in significant reduction of colonization of vaccinated birds relative to nonvaccinated birds.

Keywords: *Campylobacter*, *cjaD*, immunization, Pal

Introduction

The cell envelope of Gram-negative bacteria is comprised of inner and outer membranes, which are separated by a periplasmic space containing a rigid layer of peptidoglycan. The peptidoglycan-associated lipoprotein–tolerance protein (Pal-Tol) system, which is well conserved in Gram-negative bacteria, is responsible for maintaining cell wall integrity and is involved in many other cellular processes, such as cell division or release of outer membrane vesicles.[1,2] The proteins are generally encoded by an operon, which in *Escherichia coli*, has seven genes.[3] At least five of these genes form links between outer and inner membranes via peptidoglycan–protein and protein–protein interactions. TolQ, TolA, and TolR, which are localized in the inner membrane, create a complex interacting by their transmembrane domains. Pal is anchored by a lipid modified N-terminal in the outer membrane and associates with the β-propeller C-terminal domain of periplasm-located TolB. TolB also interacts with two outer-membrane Pal-associated proteins, Brown's lipoprotein (Lpp) and

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outer-membrane protein A (OmpA). TolA interacts with Pal in an energy-dependent manner and connects two protein complexes.[4,5] In the plant pathogen *Erwinia chrysanthemi* the *pal* gene is essential; a knockout mutant is lethal unless the mutated cells are cultivated on Luria-Bertani plates supplemented with sucrose.[6] Pal is involved in the pathogenesis of some bacterial species, such as *E. coli*,[7] *Haemophilus ducreyi*,[8] *Salmonella enterica*,[9] or *Aggregatibacter actinomycetemcomitans*.[10] The mechanism by which Pal proteins influence pathogenesis is not completely clear. Bacterial lipoproteins, being molecules described as pathogen-associated molecular patterns (PAMP), are potent modulators of the host immune system, regarding both innate and adaptive immunity. Toll-like receptors (TLR) that are present mainly on the surface of antigen-presenting cells – macrophages or dendritic cells – recognize these molecules and initiate a signaling pathway that stimulates the secretion of cytokines, such as tumor necrosis factor-α, interleukin 1 (IL-1), or IL-6. Pal exerts a pro-inflammatory effect through signaling via TLR2 and activation of nuclear factor κB.[11] The host immune system recognizes lipoprotein mainly through association of TLR2 with two other TLRs, TLR1 and TLR6. TLR1 and TLR6 are involved in the discrimination between diacyl and triacyl lipoproteins.[12,13] Some Gram-negative pathogens release lipoproteins during growth; these lipoproteins also play an important role in the induction of cytokine production. Peptidoglycan-associated lipoprotein released by diverse Gram-negative bacteria into the bloodstream is one example of this group of lipoproteins. This process plays an important role in the initiation of inflammatory responses in sepsis.[7,14] TLR2 activation by bacterial lipoproteins, such as Pal, modulates endothelial function and the coagulation pathway in multiple ways.[15]

Since Pals are an important pathogenesis factor and because they are highly immunogenic, they were tested as candidates for subunit vaccines, especially in preventing nontypeable *Haemophilus influenzae* (NTHi) infections. There is evidence that intranasal immunization with *H. influenzae* P6 (Pal) in combination with cholera toxin evokes specific immune responses and partially protects mice against infection.[16,17] Additionally, intranasal immunization of mice with P6 combined with another mucosal adjuvant (α-galactosyloceramide α – GalCer) induced high levels of specific anti-P6 nasal IgA, as well as serum IgG antibodies.[18] Thus, both of these vaccine prototypes might provide effective regimens to prevent children's respiratory tract diseases and otitis media. However, passive immunization of mice with anti-Pal monoclonal antibodies does not protect animals from sepsis.[19]

Campylobacter Pal (*Campylobacter jejuni* antigen D – CjaD), 18-kDa outer-membrane protein – Omp18 (annotated as Cj0113 in *C. jejuni* strain NCTC11168) has been identified in several labs as highly immunogenic and conserved among different *Campylobacter jejuni/coli* isolates.[20–22] It specifies an 18 kDa protein that strongly reacts with rabbit anti-*Campylobacter* antibodies, and also with convalescent sera taken from patients infected with different *C. jejuni* serotypes. Presumably, the gene product is a Pal responsible for bridging the outer membrane and peptidoglycan. Proteomics experiments proved that *Campylobacter* CjaD is a membrane-associated protein and additionally confirmed its immunogenicity in human infection.[23] Preliminary experiments conducted in our lab indicated the presence of *Campylobacter* TolB-Pal interaction, as was shown by an in vitro pull down experiment using purified rCjaD and rTolB (unpublished data). To date, all our attempts to construct a *cjaD* mutant strain by allele-exchange technology have been unsuccessful. This suggests that, like other Gram-negative bacteria, this gene may be essential (unpublished data). Genetic organization of the *Campylobacter tol-pal* genes is similar, but not identical, to the organization described for *E. coli* and many other Gram-negative bacteria.[24] Extensive BLAST searches of the *C. jejuni* NCTC11168 genome sequence revealed the presence of *tolQ*, *tolR*, *tolB*, and *pal* homologs annotated as *cj0109*, *cj0110*, *cj0112*, and *cj0013*, respectively; no ortholog of the *tolA* gene was found.[3]

The *cjaD* gene was cloned into the high-copy-number balanced-lethal plasmid and introduced into an avirulent *S. enterica* sv. Typhimurium Δ*crp* Δ*cya* strain, where it is expressed at high levels from its own promoter.[25] This study was undertaken to examine the immunogenicity of this construct in a chicken model.

Materials and methods

Bacterial strains, plasmids, media, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain BL21 (DE3) was used for overproduction of a recombinant protein. *Salmonella enterica* sv. Typhimurium strain χ3987 was employed as a host for balanced-lethal vectors and *S.* Typhimurium strain χ4172 as a source of outer membrane proteins (OMPs) used for enzyme-linked immunosorbent assays (ELISA). All *S.* Typhimurium strains were kindly provided by Roy Curtiss III (Arizona State University, Tempe, AZ).[25] *Escherichia coli* and *S.* Typhimurium strains were routinely cultured in Luria-Bertani (LB) broth (Sigma-Aldrich, St Louis, MO) or on LB

Table 1 Bacterial strains and plasmids used in this study

Name	Relevant characteristics	Source or reference
***E. coli* strains**		
BL21(DE3)	F^- *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
***S.* Typhimurium strains**		
χ3987	UK-1 Δ*crp-11* Δ[*zhc-1431*::Tn*10*] Δ*cya-12* Δ[*zid-62*::Tn*10*] Δ*asdA1* Δ[*zhf*::Tn*10*]	46
χ4172	UK-1 Δ[*fli-8007*::Tn*10*] (Fla^- Mot^- Tc^R) Δ(*galE-uvrB*)-*1005* (Bio^- Gal^- UV^s)	25
***C. coli*/*jejuni* strains**		
C. coli 72Dz/92	Wild type; isolated from a child with diarrhea	25
C. jejuni 12	Wild type; isolated from a chicken; good colonizer	This study
Plasmids		
pGEX-4T-2	Ap^R; overexpression vector	Amersham biosciences
pYA3341	Asd^+; *trc* promoter; expression vector	47
pUWM208	*cjaD* in pYA3341	25
pUWM730	*GST-cjaD* fusion in pGEX-4T-2	This study

agar plates at 37°C overnight or to a logarithmic phase of growth. Ampicillin was added to media when appropriate at the final concentration of 100 μg/mL.

The *Campylobacter coli* strain 72Dz/92, obtained from the Child Health Center, Warsaw, Poland, was used as a source of the *cjaD* (*pal*) gene. *Campylobacter jejuni* strain 12, a broiler isolate, obtained from the National Food and Nutrition Institute, Warsaw, Poland, was employed in protection experiments. Species of *Campylobacter* strains were confirmed using polymerase chain reaction with the appropriate set of primers.[26] All *Campylobacter* strains were routinely grown on Blood Agar Base No 2 (Merck KGaA, Darmstadt, Germany) plates supplemented with 5% horse blood and *Campylobacter* Selective Supplement (Blaser-Wang) (Oxoid, Basingstoke, UK) at 37°C for 16–24 hours under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). *Campylobacter* charcoal differential agar supplemented with Modified Preston *Campylobacter* Selective Supplement (Oxoid) was used to enumerate the *C. jejuni* colonies recovered from the animals.

The *C. coli* 72Dz/92 *cjaD* (*pal*) gene was identified during immunological screening of the *Campylobacter* genomic DNA library with rabbit antibodies raised against whole formalin-killed bacterial cells.[27] A DNA fragment carrying the *cjaD* gene was recloned into the high-copy balanced-lethal plasmid (pYA3341). The resulting recombinant plasmid was named pUWM208.[25] The balanced-lethal vectors, ie, the empty plasmid (pYA3341) and the pUWM208 recombinant plasmid carrying the *cjaD* (*pal*) gene, were introduced into *S.* Typhimurium strain χ3987 by electroporation.

Preparation of subcellular fractions

Periplasmic proteins were released from the cells using an osmotic-shock procedure.[28] After decanting the periplasmic fraction, bacterial pellets were resuspended in phosphate-buffered saline and sonicated to release the cell contents. Subsequently, cell wall debris was removed and the supernatants were ultracentrifuged (100,000 g, 4°C, 1 h) to separate the membrane and cytoplasmic fractions. Finally, the cell envelope was fractionated into inner and outer membranes by selective solubilization of the inner membrane with N-lauroylsarcosine sodium salt (MP Biomedicals, Solon, OH).[29] Acidic glycine extracts were prepared by the procedure of Garvis.[30]

Preparation of anti-rCjaD serum

A DNA fragment, encoding the CjaD protein without the signal sequence, was amplified from *C. jejuni* chromosomal DNA with primers cj113SalI (5′-GTCGACAAA AAGCACTAGCGTAAGCGG-3′) and cj113NotI (5′-G CGGCCGCAAAGTAGCTCCAAGAAG-3′) and cloned into pGEX-4T-2 using SalI/NotI restriction enzymes. The resulting plasmid, pUWM730, which carries a fusion of *cjaD* to *GST* at the 5′-terminus, was verified by sequencing, and then was transformed into *E. coli* strain BL21 (DE3). Bacteria were grown to a logarithmic phase of growth and the expression of the *GST-cjaD* fusion was induced with 1 mM IPTG. Bacteria were lysed by sonication and the recombinant protein was purified by affinity chromatography using glutathione-agarose (Sigma-Aldrich) following the manufacturer's protocol. The recombinant protein was extensively dialyzed against phosphate buffered saline and used for rabbit immunization.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting procedures were done by standard techniques. Blots were developed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyl phosphate (Sigma-Aldrich) as substrates, using rabbit anti-rCjaD serum as primary antibodies and mouse anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) as secondary antibodies.

Immunization experiments

Experiments were performed using commercial broiler chickens obtained from a local hatchery on the day of hatch.

A total of 69 birds were used in the study. Chickens were housed in an animal facility in separate cages for each group and given water and feed ad libitum. All studies involving animals were performed in accordance with ethical standards, after the approval from the local ethics committee.

Immunization experiments were performed as described previously.[25] Briefly, 1-day-old birds were given a primary vaccination with 10^8 colony forming units of the appropriate *Salmonella* strain by oral gavage and were given a second dose two weeks later. Every two weeks (ie, at 0, 2, 4, 6, and 8 weeks post-hatch), 15 (on the day of hatch) or 7 to 9 birds (at the other time points) were sacrificed and blood and gut samples were collected (Table 2). The levels of the antibodies against *Salmonella* OMPs and *Campylobacter* membrane proteins in chicken intestinal secretions and sera were measured by an ELISA test, as previously described, using two coating antigens: *Salmonella* OMPs and *Campylobacter* membrane-associated proteins.[25] Briefly, 96-well plates were coated with either *Salmonella* OMPs or *Campylobacter* membrane proteins, blocked with milk, and washed and incubated with diluted sera (1:100) or intestinal secretion samples (1:5). The plates were developed with 3,3′,5,5′-tetramethyl benzidine or *p*-nitrophenyl phosphate substrate (Sigma-Aldrich) using goat anti-chicken IgA Fc HRP conjugate (AbD Serotec, Kidlington, UK) and rabbit anti-chicken IgG alkaline phosphatase conjugate (Sigma-Aldrich), respectively. Optical density was determined at 450 nm or 405 nm using an ELISA reader (Labsystem Multiscan Plus; Thermo Fisher Scientific, Waltham, MA), respectively. Each sample was analyzed in triplicate.

Assessment of protection

The protection experiment was performed according to the modified procedure described previously.[25] Chickens were split into 10-bird groups and housed in separate cages. Birds were immunized with *Salmonella* strains carrying the pUWM208 plasmid, as described above. A group immunized with the *Salmonella* strain carrying the "empty" pYA3341 plasmid was used as a control. Two weeks after the booster, all the chickens were given an oral dose containing approximately 10^6 colony forming units of wild-type *C. jejuni* 12 strain in 0.1 mL of phosphate buffered saline.[31] One and 2 weeks after the challenge, five birds from each group were sacrificed and their cecal contents were aseptically removed for enumeration of *C. jejuni* colonies on *Campylobacter* charcoal differential agar plates. The scheme of the protection experiment is shown in Table 2.

Table 2 Scheme of immune response and protection experiments

Week of life	0	1	2	3	4	5	6	7	8
Immunization with *Salmonella*	+		+						
Collection of blood and gut secretion samples for immune response analysis	+		+		+		+		+
Challenge with *Campylobacter*					+				
Cecum isolation for *Campylobacter* enumeration						+	+		

Note: + indicates when the procedure occurred.

Results and discussion

CjaD localization

CjaD localization in the *C. coli* 72Dz/92 strain was analyzed by standard cell fractionation experiments. Proteins derived from different *Campylobacter* cellular compartments were analyzed by Western blot experiments using rabbit anti-rCjaD antibodies. Pal protein was present mainly in the outer membrane fraction, but it was also found in the inner membrane – even when the N-lauroylsarcosine method, recognized as the best for obtaining pure OMPs, was employed.[32] The results are shown in Figure 1. It is probable that Pal is not only anchored in the outer membrane but is also connected with the inner membrane

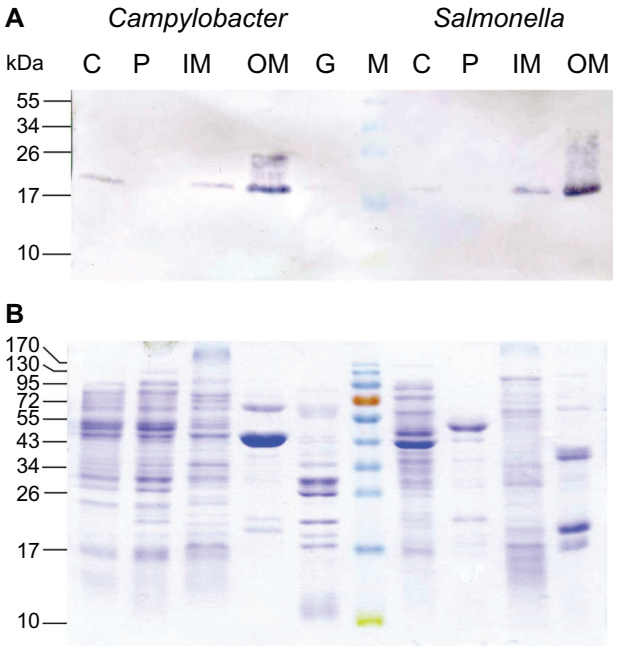

Figure 1 Subcellular localization of *Campylobacter* Pal protein in *Campylobacter* and *Salmonella* cells.

Notes: Cells were subjected to the fractionation procedure to obtain cytoplasmic (C), periplasmic (P), inner membrane (IM), and outer membrane (OM) fractions. *Campylobacter* cells were additionally subjected to acidic glycine extraction (G) (**A**) Equal amounts of protein (~10 μg) were electrophoretically separated on a 12% SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with polyclonal anti-rCjaD antibodies. Lane: M PageRuler Prestained Protein Ladder (Fermentas). The relative positions of molecular weight markers are listed on the left. (**B**) The corresponding gel stained with Coomassie blue.

through interaction with Tol, as has been documented for other Gram-negative bacteria.[4] Fractionation experiments performed on an *S. enterica* strain producing *Campylobacter* CjaD protein showed membrane localization identical to *Campylobacter,* with the CjaD protein mainly found in the outer-membrane fraction of cells. However, as was seen in the *Campylobacter* cells, a small amount of CjaD was also present in the inner-membrane fraction of cells (Figure 1).

Induction of specific systemic and mucosal immune responses

Previously, it was shown that oral chicken immunization with avirulent *Salmonella* (Δ*crp* Δ*cya*) expressing *Campylobacter cjaA* (*cj0982c*) evokes specific immune responses and that this kind of immunization can protect against colonization.[25] In this immunization study, we examined whether oral administration of the same avirulent *Salmonella* strain expressing *Campylobacter cjaD* can induce a local/mucosal and systemic immune response against *Campylobacter* membrane proteins and *Salmonella* OMPs. The group of 49 chickens was inoculated with *Salmonella*/pUWM208 (expressing *cjaD*). The schedule of immunization was identical to that used previously in the *cjaA* study, which let us compare the kinetics and the levels of specific immune response induction by two *Campylobacter* immunogenic proteins after oral vaccination.[25]

Figure 2 provides the data of the immune responses of chickens immunized with avirulent *S. enterica* χ3987 producing *C. jejuni* CjaD. The candidate vaccine prototype induced specific systemic and mucosal immune responses against OMPs of the carrier strain and against *Campylobacter* membrane proteins following the oral route of administration. The titers of anti-*Salmonella* and anti-*Campylobacter* IgG consistently increased from 2 weeks after the booster (when birds were 4 weeks old), and reached the highest values at week 8, when the experiment was terminated. High levels of anti-*Campylobacter* IgG were documented in the sera of 1-day-old birds, which confirms earlier reports that young chicks are protected against *Campylobacter* infections by maternal antibodies directed against membrane proteins.[33]

In the case of intestinal IgA, there were differences between the kinetics of anti-*Salmonella* and anti-*Campylobacter* IgA induction. The first peaked 2 weeks after the second immunization (when birds were 4 weeks old). But the latter increased at 2 weeks and reached the highest value 6 weeks after the booster. This result is in contrast to that observed when birds were immunized with *Salmonella*/pUWM251 (expressing *cjaA*)[25] – in that experiment the kinetics of IgA responses to both antigens were similar. In the case of chicks immunized with *Salmonella* expressing *cjaA*, the level of IgA antibodies to *Salmonella* and *Campylobacter* antigens present in the intestinal secretions peaked two weeks after the booster. In this study, we did not evaluate the viability of the carrier strain in the ceca of birds. We can only speculate that various heterologous antigens (*cjaA* vs *cjaD*) expressed by the *Salmonella* strain influence its viability in the host, and this may lead to differences in the kinetics of the immune responses. In contrast

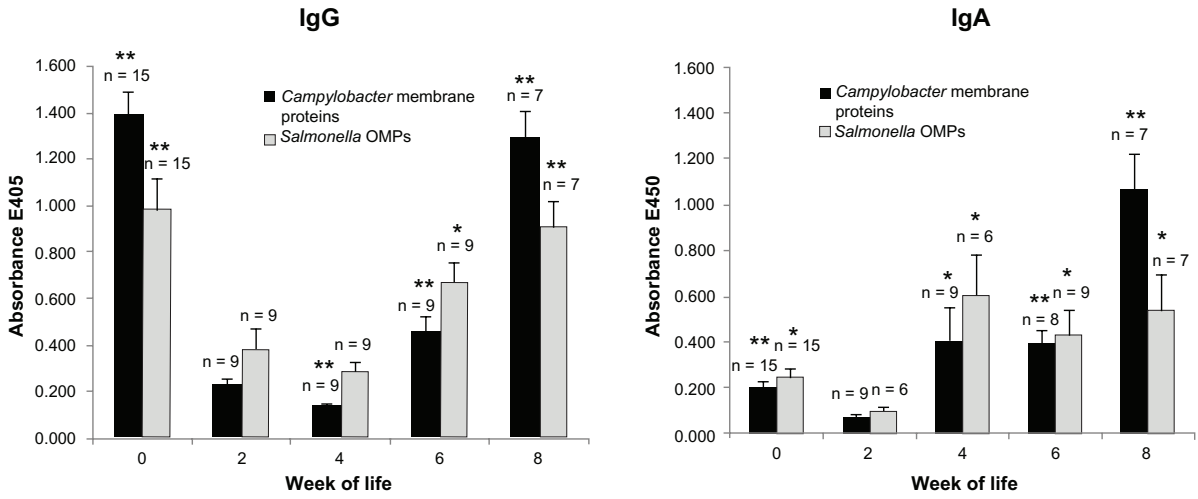

Figure 2 ELISA analysis of the immune responses of chickens immunized with avirulent *S. enterica* χ3987 producing *C. jejuni* Pal protein.
Notes: The two doses were given orally to chickens on days 1 and 14. Serum and intestinal samples (ceca) were collected when birds were 0, 2, 4, 6, and 8 weeks old. *Salmonella* OMPs and *Campylobacter* membrane proteins were used as coating antigens. Serum samples were diluted 1:100 and intestinal secretion samples 1:5. Error bars indicate standard error of the mean. An asterisk indicates that the value is significantly different compared with the value for 2-week-old birds (*$P < 0.05$; **$P < 0.005$; two-tailed Student's *t*-test).
Abbreviation: OMPs, outer membrane proteins.

to specific IgGs, a low level of anti-*Campylobacter* outer-membrane protein IgA was observed in the intestinal secretion of 1-day-old chicks (Figure 2). It was slightly higher than that present in the intestinal secretions of 2-week-old birds, suggesting that these IgAs represent maternal antibodies that might participate in chicken protection against *Campylobacter* during the first 2 weeks of life.

Protection experiment

The vast majority of *Campylobacter* infections in developed countries occur as sporadic cases, and consumption of undercooked poultry meat is a key risk factor of human campylobacteriosis.[34,35] Although many *Campylobacter* whole cell and subunit vaccine prototypes have been constructed and tested within the last 10 years, no effective chicken anti-*Campylobacter* vaccine is available on the market.[36–40]

Protection experiments were carried out to test the effectiveness of attenuated *S. enterica* sv. Typhimurium to protect the chicken gut against colonization by *C. jejuni.* In total, 10 vaccinated and 10 nonvaccinated chickens were challenged with 10^6 bacterial cells of a broiler-isolated *C. jejuni* strain 2 weeks after the booster and examined for *Campylobacter* colonization 7 and 14 days later. Challenge experiments were preceded by studies showing that chicks were not already colonized by *Campylobacter.* The results of the protection study are summarized in Figure 3. Protection was not observed using a vaccine strain containing the empty plasmid. All the birds' ceca became colonized with *C. jejuni,* reaching mean concentrations of about 1×10^9 per g of cecal contents. The same level of colonization was observed for nonimmunized birds (data not shown). Furthermore, vaccination with *S. enterica* expressing CjaD had no significant effect on the cecal load of *C. jejuni* relative to the control. However, it was observed that the levels of colonization between vaccinated birds differed significantly, especially 2 weeks after the challenge. Further experiments are needed to explain this fact. The observed discrepancies might be explained by differences in the host response to *C. jejuni* infection at a molecular level. Recently conducted comprehensive transcriptomic analysis of spleen and ceca RNA from two genetically distinct lines of broiler chickens revealed significant differences in response to *C. jejuni* infection. Interestingly, the differences in gene expressions were observed not only between two lines of chickens but also between birds within each line.[41–43]

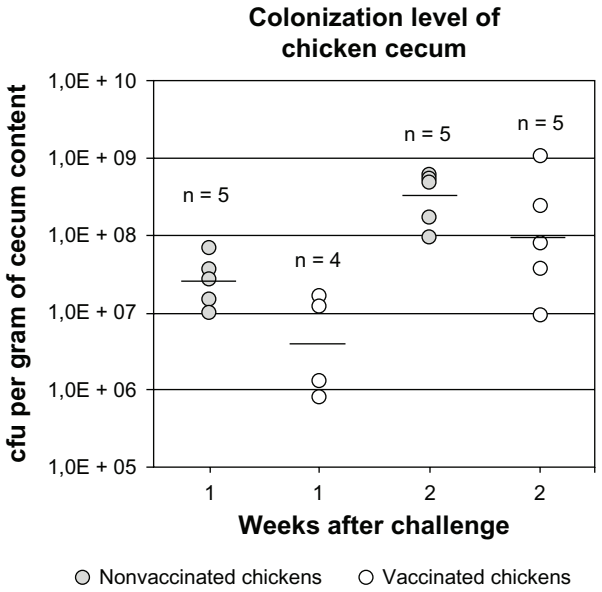

Figure 3 Assessment of protection.
Notes: Colonization of vaccinated and nonvaccinated chickens 1 and 2 weeks after the challenge with wild-type *C. jejuni* (inoculation dose 10^6 cfu). A bar indicates a geometric mean value. In one case, the colonization level of a vaccinated chicken was not determined due to a technician's error. The difference between level of colonization is statistically significant only one week after challenge (*P*-value = 0.05; two-tailed Student *t*-test).
Abbreviation: cfu, colony forming units.

Recently, Layton et al used attenuated *S. enterica* sv. Enteritidis expressing Pal linear epitope as a fusion with LamB protein for chicken vaccination.[38] They showed that one-time immunization with this construct, coexpressing the immune-enhancing CD154 ligand that plays a role in the regulation of a cellular immune response, reduced colonization of immunized birds by wild-type *C. jejuni* (4.8 log reduction of *C. jejuni* compared to the control). The mechanism underlying this excellent protection is unknown. However, there were many key differences between Layton's study and our own, such as: the chicken lines used for vaccination, the immunization schedule, the vector strain, the coating antigen for the ELISA assay, and the techniques used for protection assessment. To enumerate *C. jejuni* colonization, Layton et al employed quantitative real-time polymerase chain reaction on DNA extracted from the mucosal lining of the ileum and did not confirm the data by standard plating methods.

When Layton et al used their own strategy to immunize birds with CjaA antigen, they observed only a two-log reduction of *Campylobacter* colonization after vaccination, whereas using *S. enterica* sv. Typhimurium χ3987 expressing CjaA for bird immunization caused reduction of colonization by a heterologous *C. jejuni* strain by six logs as compared to a nonimmunized control.[25,38] In a different study, immunization of specific pathogen free Light Sussex chickens with *S. enterica* sv. Typhimurium expressing CjaA fusion to the C-terminus of tetanus toxin resulted in

reduction of colonization of the chicken intestinal track by 1.4 logs compared to the untreated control.[36,38]

Both examples indicate that the data derived from studies performed by different research groups are not consistent and that these discrepancies may have different sources. Experts in the field emphasize that there is an urgent need to control chicken contamination by *Campylobacter* and agree that close cooperation between research groups is needed, especially to work out specific protocols for vaccine efficiency evaluation.[44,45]

In spite of the limited protective response generated against *Campylobacter* colonization by immunization with *Salmonella* χ3987 expressing the *Campylobacter cjaD* gene, it is reasonable to propose further study aimed at using CjaD protein as an element of a multicomponent chicken subunit anti-*Campylobacter* vaccine. As documented in this study, our method of immunization induced significant immune responses. Thus, further modifications of the *Salmonella* vaccine strain expressing *cjaD* or its epitopes by manipulation of the amount and the localization of the antigen might enhance its efficacy.

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Disclosure

The authors report no conflicts of interest in this work.

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