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Application of a liposomal subunit vaccine in chickens for reduction of *Campylobacter* gut colonisation

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Abstract

Introduction: *Campylobacter* are the most common cause of food poisoning, which manifests itself in diarrhoea of varying severity. Additionally, because of the increasing number of people with immune deficiencies, more frequent serious complications of *Campylobacter* infections are being observed. The main source of infection is the consumption of contaminated poultry meat, which is a consequence of the insufficiency of current hygiene and biosecurity to control *Campylobacter* or eliminate it from the poultry food chain. **Material and Methods:** Two hybrid proteins, presenting selected epitopes of the *Campylobacter* antigens CjaD and EF-Tu, were developed based on the highly immunogenic proteins CjaA and CjaC. Four groups of chickens were vaccinated with different preparations (a mixture of both hybrid proteins encapsulated in anionic or neutral liposomes) and different doses (a single dose given on the day of hatching or two doses given on days 1 and 14 of life). The number of *Campylobacter* was assessed in the intestinal contents of vaccinated birds. **Results:** No statistically significant differences in colonisation levels were observed between chickens immunised with neutral liposomes containing hybrid proteins and their non-immunised counterparts, regardless of dosage regimen. **Conclusion:** Although immunisation of chickens did not produce the expected results, the approach used has great potential, which is worth further investigation and development.

Keywords: *Campylobacter*, vaccine, liposomes, hybrid proteins.

Introduction

Campylobacter, microaerophilic, spiral-shaped, Gram-negative microorganisms belonging to the Epsilonproteobacteria class, are the aetiological agent of campylobacteriosis, the most diagnosed zoonosis among residents of European Union member states since 2005. According to the European Food Safety Authority (EFSA), there were 137,107 confirmed cases of *Campylobacter* infections in 2022 (9). Recently, the incidence rate has remained relatively stable with a slight downward trend, ranging from 58.3 cases per 100,000 people in 2018 to 43.1 cases per 100,000 people in 2022 (9).

Campylobacter infection usually has a low mortality rate, and most patients do not require specific therapy. Antibiotics (macrolides and fluoroquinolones) are used only in severe systemic infections or in patients with impaired immunity (27). However, epidemiological data have recorded cases of serious autoimmune and neurological complications that develop following *Campylobacter* infections. One such example is neuropathy of the peripheral nervous system, known as Guillain-Barré syndrome (GBS) (11). Current research also indicates a connection between *Campylobacter* infection and the development of reactive arthritis, inflammatory bowel disease, irritable bowel syndrome, and colorectal cancer (22). It is estimated that for every reported case of campylobacteriosis, there are 30 more cases that go undiagnosed because of the mildness of the usual symptoms of infection.

Epidemiological studies demonstrate that the majority of human campylobacteriosis cases are primarily caused by the consumption of undercooked infected poultry meat. This aligns with the observation that farmed poultry is the main reservoir of *Campylobacter* (36). These microorganisms colonise the birds' digestive systems at exceptionally high levels (up to 10^9 colony-forming units (CFU)/g of intestinal contents), contributing

to meat contamination during the production process. Most of the studies conducted to date indicated that high levels of chicken gut colonisation do not cause clinical symptoms in birds, thereby vitiating the isolation of infected individuals from the flock. However, there are also reports describing adverse health effects of *Campylobacter* colonisation on chicken intestines (1).

On January 1, 2018, the EU Commission Regulation No. 2017/1495 introducing a hygiene criterion for *Campylobacter* in broiler carcasses came into force. This regulation stipulated that the allowable number of CFU/g of poultry meat should not exceed 1,000. It was assumed that decreasing the population of *Campylobacter* in the gastrointestinal tract of chickens would significantly reduce the incidence of human campylobacteriosis, and consequently, would lower healthcare costs. According to the updated model estimation, a reduction of 3 $\log_{10}$ could result in a 58% decrease in the risk of *Campylobacter* infection associated with the consumption of contaminated poultry meat (10). In 2022, an EFSA report indicated that approximately 38% of examined broiler carcasses were contaminated with *Campylobacter* (9).

One of the promising approaches to reducing the number of *Campylobacter* in chicken digestive systems is immunoprotection. Over the past twenty years, many attempts have been made to develop an anti-*Campylobacter* vaccine for chickens with varying degrees of success, including whole-cell, DNA, carrier-based and subunit vaccines. Data on this topic have been collected recently in some review papers (30, 31). Many studies have indicated that vaccine formulations based on a single antigen exhibit inadequate protective potential. In our previous study, a recombinant rCjaAD protein was created. It is composed of *Campylobacter jejuni* antigen A protein (CjaA) which presented three selected *Campylobacter jejuni* antigen D (CjaD) epitopes on its surface. Administration of rCjaAD to chickens, either encapsulated in liposomes or using a carrier strain of lactic acid bacteria, resulted in a reduction of *Campylobacter* in the intestines by approximately 2 $\log_{10}$ (24, 25). Hence, in this study, a subunit vaccine for poultry was developed, composed of a mixture of four *Campylobacter* antigens in the form of two hybrid proteins. We used two immunogenic *Campylobacter* proteins, CjaA and CjaC (*Campylobacter jejuni* antigen C), as the "backbone", and two others – CjaD and EF-Tu (elongation factor thermo unstable), as sources of epitopes, resulting in rCjaAEF-Tu and rCjaCD.

The "backbone" components are glycosylated extracytoplasmic lipoproteins. They belong to the substrate-binding adenosine triphosphate–binding (ABC) cassette transport system and are conserved among various *Campylobacter* serotypes. Crystallographic analyses of rCjaA have indicated that this protein may be responsible for cysteine transport; however, growth experiments have suggested that other amino acids may also be involved (40). Conversely, histidine is the most probable ligand for CjaC (12). Amino acids constitute the crucial elements of *Campylobacter* metabolism as they serve not only as a nitrogen source but also as carbon and energy supplies. Therefore, proteins involved in amino acid transport play a significant role in many important physiological processes in this microorganism. Additionally, the expression of the *cjaA* gene is upregulated in iron-deficient media, suggesting the involvement of CjaA in colonisation processes *in vivo* (20). It has also been shown that both the CjaA and CjaC proteins occur in higher quantities in cells of fresh clinical isolates than in cells of repeatedly passaged laboratory strains (8). Moreover, they are recognised by maternal antibodies, which protect chicks from *Campylobacter* during the first weeks of life (8, 35).

The CjaA and CjaC "backbone" proteins were further modified by adding epitopes derived from EF-Tu and CjaD proteins, respectively. Numerous recent studies have shown that EF-Tu may be implicated in bacterial pathogenesis in addition to its involvement in protein synthesis (17). This protein is recognised by secretory immunoglobulin A (IgA) in one-week-old chicks and is responsible for inducing specific IgY during *Campylobacter* infection (19). The peptidoglycan-associated lipoprotein CjaD, anchored to the outer membrane of bacterial cells, was proved to be highly immunogenic in many pathogens (15).

A subunit vaccine of an assortment of *Campylobacter* antigens carried in two types of liposomes was administered to chicks to assess the protective potential of the antigens. The study also expanded the body of knowledge on liposome multi-antigen vaccines.

Material and Methods

Bacterial strains and standard genetic manipulations. *Escherichia coli* TG1 was used as a host for the construction of recombinant plasmids. Strain BL21 (with the DE3 lysogen) and *E. coli* OverExpress C43 (DE3) (Sigma-Aldrich, St. Louis, MO, USA) were used to produce rCjaCD and rCjaAEF-Tu, respectively. The bacteria strains were grown at 37°C in lysogeny broth medium or ZYP-0.8G medium followed by ZYP-5052 medium for auto-induction of recombinant proteins supplemented with 100 μg/mL ampicillin (37).

The *C. jejuni* 12/2 used to challenge birds in animal experiments was a broiler-isolated strain labelled with the pUOA18 plasmid containing a *cat* gene. The 12/2 strain was cultured under microaerobic conditions at 42°C on Blood Agar Base medium (Oxoid, Basingstoke, UK) supplemented with 5% horse blood, 15 μg/mL chloramphenicol and Blaser–Wang Campylobacter Selective Supplement (Oxoid). Common genetic manipulation procedures were performed according to the standard protocols of Sambrook and Russell (16).

Prediction of epitopes and protein structures. The analysis of the protein structures of CjaA (CJJ81176_1001) and CjaD (CJJ81176_0148, Omp18) described earlier was utilised (24). Modelling of the structure of the EF-Tu (Cjj81176_0499), CjaC (Cjj81176_0757, HisJ), rCjaAEF-Tu and rCjaCD hybrid

proteins was carried out using the secondary and tertiary structure prediction programs Quick2D and RaptorX (which builds its models relying on the templates available in Research Collaboratory for Structure Bioinformatics Protein Data Bank; RCSB PDB). Epitopes were predicted from amino acid sequences using the following methods: Emini Surface Accessibility Prediction; ElliPro: Antibody Epitope Prediction, Kolaskar and Tongaonkar Antigenicity; and BepiPred Linear Epitope Prediction (24). All results were compared, and consensus predictions were mapped to homology models of the CjaA and CjaC proteins. Consensus fragments located in loops exposed to the solvent were considered the most probable epitopes. Modelling was performed based on available structures in RCSB PDB. The best matrix for rCjaAEF-Tu is RCSB PDB - 1XT8: Crystal Structure of Cysteine-Binding Protein from *Campylobacter jejuni* at 2.0 A Resolution, and the best matrix for rCjaCD is RCSB PDB - 4ZV1: An ancestral arginine-binding protein bound to arginine and RCSB PDB - 4YMX: Crystal structure of the substrate binding protein of an amino acid ABC transporter.

Recombinant plasmids constructed in this study. The r*cjaAEF-Tu* and r*cjaCD* genes encoding the hybrid proteins were synthesised by GeneCust (Boynes, France) and cloned into the pBluescript (SK)+ vector (Stratagene, now Agilent Technologies, La Jolla, CA, USA) of 3 kb length with ampicillin resistance, resulting in the plasmids designated pUWM1531 and pUWM1529, respectively. Subsequently, using the BamHI and SalI restriction enzymes, fragments were cloned into the pET22b expression vector (Sigma-Aldrich) digested with the same endonucleases. This allowed the insertion of sequences encoding hybrid proteins between the signal sequence of the PelB protein and a 6×His tag at the C-terminus end. Both constructs were sequenced and subsequently named pUWM1536 (r*cjaAEF-Tu*) and pUWM1555 (r*cjaCD*). They provided periplasmic localisation of the hybrid proteins. The pUWM1536 and pUWM1555 plasmids were transformed into strains that allowed expression from pET-type vectors.

Expression and purification of recombinant rCjaCD and rCjaAEF-Tu proteins. The rCjaAEF-Tu and rCjaCD proteins were overexpressed by auto-induction and purified by Ni-NTA affinity chromatography from *E. coli* OverExpress C43 (DE3) and *E. coli* BL21 (DE3) strains (harbouring pUWM1536 and pUWM1555, respectively), as described by Studier (37). The obtained proteins were used to generate specific rabbit sera (anti-rCjaCD and anti-rCjaAEF-Tu) and for chicken vaccination in a protective experiment. Western blotting and SDS-PAGE procedures were performed by standard techniques. Blots were developed with NBT/BCIP (Sigma-Aldrich) as a substrate, using the rabbit polyclonal anti-rCjaCD and anti-rCjaAEF-Tu obtained in the present experiment; anti-CjaA, anti-CjaC, anti-CjaD and anti-EF-Tu (29); or anti-His (6× His Tag Monoclonal Antibody MA1-135; Invitrogen, Carlsbad, CA, USA) sera as primary antibodies, and mouse anti-rabbit IgG alkaline phosphatase conjugate or goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich) as secondary antibodies.

Preparation of specific antisera. Polyclonal rabbit antibodies recognising hybrid proteins were prepared by Kaneka Eurogentec (Seraing, Belgium) according to a shortened 28-day vaccination schedule. The purified rCjaAEF-Tu and CjaCD hybrid proteins were used in phosphate-buffered saline (PBS; 1 mg/mL) as antigens.

Preparation of liposomes containing rCjaCD and rCjaAEF-Tu proteins. The liposomes to contain the recombinant proteins, composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine/cholesterol/1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DPPC/Chol/DSPE-PEG2000 – 5.8:4:0.2 mol/mol) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine/1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol/ cholesterol/1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPC/DPPG/Chol/DSPE-PEG2000 – 3.8:2:4:0.2 mol/mol), were prepared using an extrusion protocol described earlier (25). Subsequently, 1.2 mL of liposomal suspension (DPPC/Chol/DSPE-PEG2000 or DPPC/DPPG/Chol/ DSPE-PEG 2000) was mixed with 1.64 mL of the rCjaCD protein solution (3.66 mg/mL) or 2.35 mL of rCjaAEF-Tu protein solution (2.55 mg/mL) and incubated for 10 min at 37°C. During incubation, the protein was incorporated into the liposome bilayer, which was visible by changing the degree of light scattering of the liposome samples without their size changing for 1 or 2 s after the protein solution was added. The free proteins were not removed from liposomal suspension prior to vaccination. The incorporation efficiency for rCjaCD was in the range of 78.58–91.32%, and for rCjaAEF-Tu protein it was in the slightly lower range of 75.64–83.71%. Liposomes were generally in the size range 260–320 nm.

Immunisation and challenge regimens. All animal experiments were conducted according to ethical standards and with the approval No. 1218/2021 of Local Ethics Committee No. 1, Warsaw, Poland. The experiments were carried out on Hy-line chicks obtained on the day of hatching from a local hatchery. The chicks were confirmed to be culture-negative for *Campylobacter* by cloacal swabbing and were subsequently randomly assigned to experimental groups, which were housed in separate cages.

The experimental setup included four groups, which differed in which orally administered preparation they received (a mixture of 50 μg of each hybrid protein encapsulated in the anionic liposome (DPPC/DPPG/Chol/DSPE-PEG 2000) or this mixture encapsulated in the neutral liposome (DPPC/Chol/ DSPE-PEG 2000)) and the number of doses they were administered (a single dose given on the day of hatching or two doses given on days 1 and 14 of life). Each group comprised 14 individuals. Following vaccination, the chicks were observed for the development of diarrhoea and other potential adverse symptoms. Two control groups of the same number of birds were administered

buffered saline with 1% gelatine, once or twice. On the 21st day of life, the birds were orally challenged with approximately 3.5 × 10^5 CFU of *C. jejuni* 12/2. On the 6th and 12th days post-infection, half of the birds from each group were euthanised, and the number of *Campylobacter* was determined in the intestinal contents. The caecal contents were diluted in PBS and plated on blood agar medium containing the appropriate supplements. The plates were incubated at 42°C for 48 h under microaerobic conditions (85% N_2, 10% CO_2 and 5% O_2). Statistical analyses of the colonisation results were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). The significance of the differences between the obtained values was assessed using the Kruskal–Wallis test, with statistical significance being set at P-value < 0.05.

Results

Protein structure prediction and epitope search. Structure modelling performed earlier for the CjaA protein resulted in the prediction of six regions appropriate for the insertion of foreign oligopeptides (here epitopes of EF-Tu): D_{25}/S_{26}, V_{88}/E_{89}, R_{189}/G_{190}, H_{207}/P_{208}, and G_{218}/N_{219} (24). An analogous search was conducted for CjaC resulting in the determination of the probable location of secondary structure elements (alpha-helices and beta-sheets), regions of disorder showing significant flexibility of the structure, and positioning of amino acid residues accessible to the solvent. This analysis identified sites suitable for inserting short oligopeptides, ensuring no disruption to the protein structure while facilitating the presentation of foreign epitopes (here of CjaD) on its surface. These regions are: D_{22}/S_{23}, N_{48}/S_{49}, T_{77}/N_{78}, N_{177}/K_{178}, and E_{205}/T_{206}.

Next, we identified epitopes that were used for the construction of hybrid proteins rCjaAEF-Tu and rCjaCD. Four different methods were applied to predict EF-Tu and CjaD fragments potentially recognised by antibodies (Material and Methods section). Then, the indicated amino acid sequences were superimposed on the backbone protein structures, and only those located on the surface and accessible to the solvent were accepted as proper epitopes. For the translation elongation factor, we first identified four oligoamino acids. However, the final choice was further refined by searching for unique sequences for *Campylobacter coli* and *Campylobacter jejuni* compared with other chicken gut microbiota. It was necessary to prevent the immune response to EF-Tu proteins of beneficial bacteria, such as members of the *Clostridiaceae*, *Bacteroidaceae*, *Ruminococcaceae*, *Lactobacillaceae* and *Lachnospiraceae* families. We found two low-homology regions in *Campylobacter* EF-Tu, which encompassed two predicted epitopes: EpT1: 37-SRRGLAELKDYDN-49 and EpT3: 181-EAKAGQDGEWSAK-193. In the case of the CjaD protein, we selected five epitopes: EpD1: 20-STKSTSVSGDSSVDSNRGSGGSDGWD-45, EpD2: 63-DFDKFNIRP-71, EpD3: 87-EVSGV-91, EpD4: 100-DEWGTDEYN-108 and EpD5: 139-GETNPVCTEKTKACDAQNRR-158.

Furthermore, in the same manner, we identified epitopes of the backbone proteins. The most promising epitopes of CjaA were previously predicted in our study (24) and are as follows: EpA1: 55-VDEKGN-60, EpA2: 79-DENKV-82, EpA3: 111-QTPERAEQ-118, EpA4: 136-KDSNITSVEDLKDK-149, EpA5: 167-YPNIK-171, EpA6: 263-FGDDVK-268. *In silico* analysis of the CjaC protein sequence indicated four regions as the most probable epitopes: EpC1: 24-KNKESNASV-32, EpC2: 43-KPFNYKENSK-52, EpC3: 101-TDERRQ-106 and EpC4: 125-KNNDSLQTKNDL-136. Assessing their localisation enabled the proper insertion of new epitopes from EF-Tu and CjaD, preventing the destruction of those already existing in the backbone proteins.

Based on the above predictions, different amino acid sequences of the CjaA protein containing EF-Tu epitopes and the CjaC protein with incorporated CjaD epitopes were designed. The structure of the rCjaAEF-Tu hybrid protein appeared stable, the core was intact, and the added epitopes were located on the surface only when the EpT3 and EpT1 epitopes were inserted at the R_{189}/G_{190} and H_{207}/P_{208} sites, respectively. In the case of the hybrid rCjaCD protein, a similar effect (meeting the above criteria) was obtained when EpD3, EpD4 and EpD2 were inserted into sites D_{22}/S_{23}, N_{48}/S_{49} and N_{177}/K_{178}, respectively. The amino acid sequences of the rCjaAEF-Tu and rCjaCD hybrid proteins and their structural models are shown in Fig. 1.

Evaluation of immunogenicity and antigenicity of hybrid proteins. Western blot analysis confirmed that the proteins rCjaAEF-Tu (approximately 37 kDa) and rCjaCD (approximately 34 kDa) reacted with specific polyvalent sera – respectively anti-CjaA and anti-EF-Tu and anti-CjaC and anti-CjaD (Figs 2A and 2B). The rCjaCD protein was purified into three main forms that differed in size, most likely because of the partial breakdown of the hybrid protein. Two of these were very close to each other and corresponded to the correct size of rCjaCD (approximately 35 kDa), whereas the third was smaller (approximately 30 kDa). All were recognised by specific antibodies; therefore, it was assumed that they could be used for further experiments. Both hybrid proteins were also recognised by maternal antibodies present in the sera of 7-day-old chicks (data not shown).

Rabbit immunisation with rCjaAEF-Tu and rCjaCD provided a means to confirm that the hybrid proteins induced specific immune responses in immunised animals, and the anticipated anti-rCjaAEF and anti-rChaCD sera were obtained. Both sera recognised the native backbone proteins as well as those of which the epitopes were used to create the hybrids. The serum of rCjaAEF-Tu-immunised animals recognised the CjaA and EF-Tu proteins in the *C. jejuni* cell lysate, and the serum of rCjaCD-immunised animals recognised both the CjaC and CjaD proteins present in the *C. jejuni* cell lysate (Figs 2C and 2D).

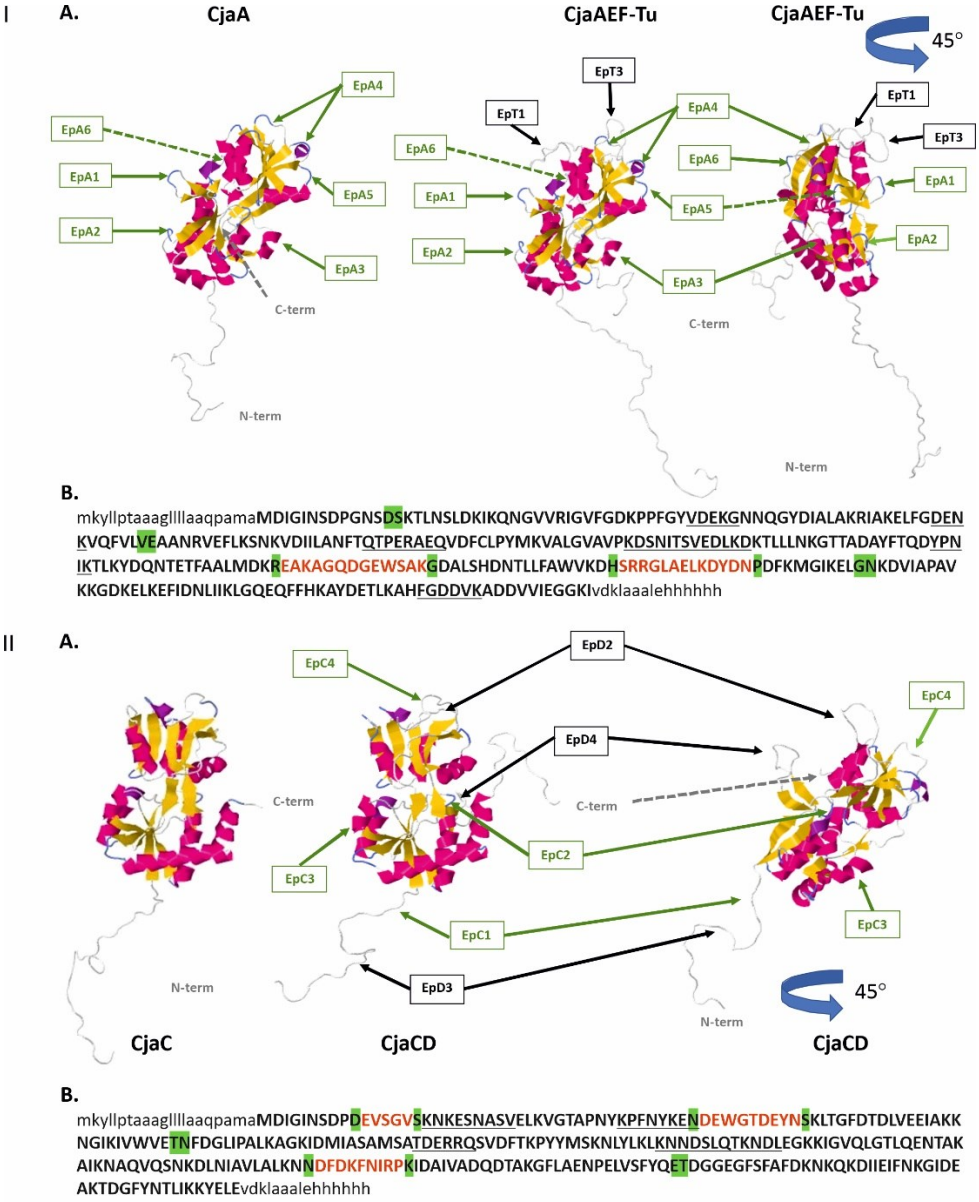

Fig. 1. A. RaptorX structure modelling for rCjaAEF-Tu (IA) and for rCjaCD (IIA). Modelling of the CjaA (IA) and CjaC (IIA) proteins with their native signal sequence is included for comparison. Protein epitopes (Ep) are shown in green (CjaA and CjaC) and black (EF-Tu and CjaD). Dashed arrows indicate elements not visible on selected orientation of protein molecule. B. The amino acid sequence of the rCjaAEF-Tu (IB) and rCjaCD (IIB) hybrid proteins. Lowercase letters denote the signal sequence at the amino terminus of the protein and the fragment at the carboxyl terminus covering the histidine tag. Epitopes of the CjaA (IB) and CjaC (IIB) proteins are indicated by underlining, epitopes of the EF-Tu (IB) and CjaD (IIB) proteins are marked in red, and insertion sites are highlighted in green

Development of strategies for packing hybrid proteins into the liposomes. Determination of the protein encapsulation efficiency after mixing empty liposomes with DPPC/Chol/DSPE-PEG 2000 with the rCjaCD protein solution showed that it exceeded 90%, which surpassed the theoretical possibilities of this method. Subsequent studies confirmed that the protein interacts with the lipid bilayer, which suggested the possibility of simplifying the encapsulation/incorporation method. After empty liposomes were mixed with protein solution and incubated at 37°C, high incorporation efficiencies were calculated for most of the formulations. In the case of the rCjaAEF-Tu protein, its interaction with the lipid bilayer was also observed, but probably with a somewhat lower binding constant. The lowest rCjaCD protein incorporation efficiency was measured for the formulation with a rigid bilayer composed of hydrogenated soy lecithin (HSPC, 43%, results not shown) and for the liquid bilayer made of soy lecithin (SPC, 55%). For the rCjaAEF-Tu protein, higher incorporation efficiency was observed for liposomes containing lipids with high phase transition temperatures (liposomes with a rigid bilayer composed of HSPC, 90%). For cationic formulations, the encapsulation efficiency of both hybrid proteins was very low (3–6%, data not shown). The DPPC lipid was chosen as the main formulation lipid because it gave high incorporation efficiencies and high protein immunisation in our previous research (25).

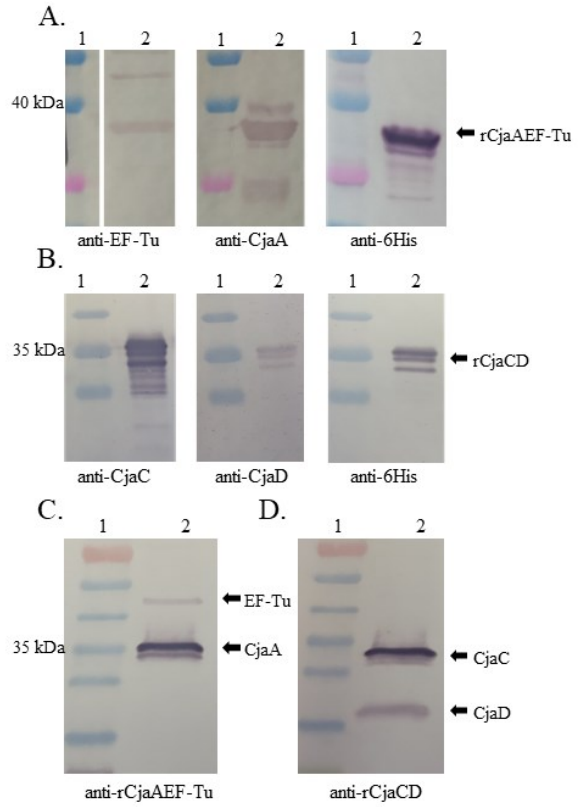

Fig. 2. Immunoreactivity and immunogenicity of hybrid rCjaAEF-Tu and rCjaCD proteins analysed by Western blot. A – Immunoreactivity of rCjaAEF-Tu with specific single antigen sera. Lane 1 – marker; lane 2 – purified rCjaAEF-Tu protein; B – Immunoreactivity of rCjaCD with specific single-antigen sera. Lane 1 – marker; lane 2 – purified rCjaCD protein; C – Immunogenicity of rCjaAEF-Tu detected with rabbit serum raised against whole hybrid protein. Lane 1 – marker; lane 2 – protein lysate from whole *C. jejuni* cells; D – Immunogenicity of rCjaCD detected with rabbit serum raised against whole hybrid protein. Lane 1 – marker; lane 2 – protein lysate from whole *C. jejuni* cells

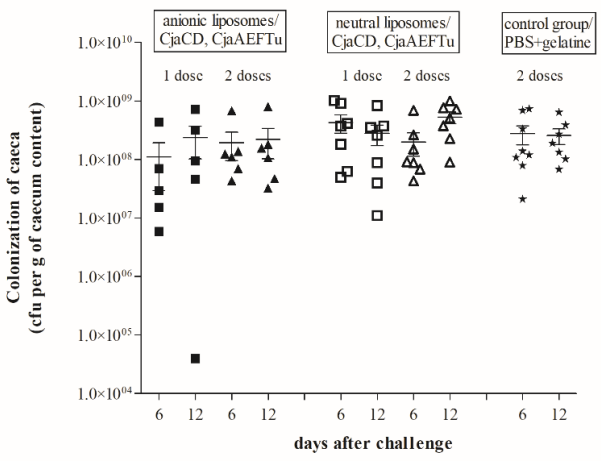

Fig. 3. Colonisation of chickens vaccinated with neutral or anionic liposomes containing hybrid proteins, rCjaCD and rCjaAEF-Tu, and of unvaccinated chickens challenged with strain 12/2 of *Campylobacter jejuni*. Viable *C. jejuni* cells recovered from the caeca of chickens were assessed on the specified days after the challenge. No significant differences (P-value < 0.05) were observed between groups. PBS – phosphate-buffered saline

Analysis of the protection afforded by chicken immunisation. Details of the vaccination procedure can be found in the Material and Methods section. On the 21st day of life, the chickens were challenged with *C. jejuni* 12/2 strain (3.5×10^5 CFU). The isolated strain colonised the intestines of chickens well and was shown to harbour a stably maintained plasmid marked with a gene conferring resistance to chloramphenicol. Figure 3 illustrates a comparison of caecum colonisation levels between vaccinated and unvaccinated birds.

No differences in colonisation levels were observed between chickens immunised with neutral liposomes containing hybrid proteins and their non-immunised counterparts, regardless of dosage regimen. A slight decrease compared to that in the control group, although not statistically significant, was noted in the group of chickens administered anionic liposomes containing rCjaCD and rCjaAEF-Tu. Within this cohort, one chicken from the single-dose vaccine group exhibited a colonisation level below 1×10^5 CFU/g of gut contents. The appearance of the caeca in some chickens in this group was notably different, these being noticeably smaller and devoid of most content. Nevertheless, smallness and near-emptiness did not correlate with the decreased levels of *Campylobacter* colonisation.

Discussion

High levels of colonisation of chicken intestines by *Campylobacter* do not cause disease symptoms in birds and do not affect the efficiency of breeding or the yield of meat. This has led to a lack of interest from the poultry industry in developing a vaccine for chickens against these pathogens. However, the increasing number of cases of campylobacteriosis in humans, complications after infection, and the growing number of antibiotic-resistant strains of *Campylobacter* have prompted the search for effective strategies to prevent *Campylobacter* infections.

One approach is to vaccinate people in high-risk groups, such as children under the age of five. The presence of antibodies in people who have suffered infection and the lack of disease in adults in endemic areas indicate that while developing a vaccine for humans is not an easy task, it is feasible. Difficulties arise from, among other things, an incomplete understanding of the pathogenesis of the disease agent and the great diversity of *Campylobacter* strains. The ability of *Campylobacter* to cause GBS also raises serious concerns about the safety of vaccines, especially those based on killed or attenuated strains of the microorganism. So far, only one formulation has reached the clinical trial stage. It is a vaccine using the capsule polysaccharides as antigens (US National Library of Medicine ClinicalTrials.gov identifier NCT02067676).

Another solution that could help reduce human infections is to decrease the level of chicken intestinal colonisation by human-pathogenic strains of *Campylobacter* spp. Various attempts have been made to control the spread of infections in chickens under mass-rearing conditions. However, measures to improve farm hygiene and biosecurity, such as the use of competitive exclusion or feed or drinking water additives (*e.g.* organic and fatty acids, plant-derived products, bacteriocins, bacteriophages, biofilm formation inhibitors or quorum-sensing inhibitors) are not sufficient to effectively control *Campylobacter* or eliminate it from the poultry food chain. It appears that the most effective method of anti-*Campylobacter* prophylaxis may be immunisation of chickens. However, despite intensive research to develop a prototype of such a vaccine, still no vaccine is commercially available.

To date, the question of what type of immune response would effectively protect chickens from *Campylobacter* colonisation has not been answered. Sahin *et al.* (33) demonstrated that maternal *Campylobacter*-specific antibodies played a role in protecting young chickens from colonisation, but this protection was short-lived and did not cover the entire period of broiler production. This study nevertheless indicates that boosting immunity may be a rational and feasible way to control *Campylobacter* in livestock farming. The relevance of the humoral immune response in preventing *Campylobacter* colonisation of chickens was confirmed by Hermans *et al.* (19). Their study showed that passive immunisation, which involved administering IgY obtained from *Campylobacter*-infected hens, significantly reduced the number of cells of these microorganisms in the intestines and prevented transmission of the pathogen between birds. Furthermore, recent research suggests that antimicrobial peptides and gut microbiota composition may play a crucial role in this process (13).

Numerous vaccination attempts have been made so far using various methods (31). Among recent trials, the utilisation of live, attenuated *Campylobacter* strains is noteworthy. Those strains carried mutations in the *ahpC* or *katA* oxidative stress defence genes, and when administered to chickens, they notably decreased colonisation by wild *Campylobacter* strains. Nevertheless, they were unable to permanently colonise the intestines of birds and could not survive in aerobic environments because their sensitivity to oxygen was greater (21).

Attention is also given to subunit vaccines, as they are generally regarded as safer than live, attenuated kinds and are easy to produce or modify. To date, several potentially useful proteins have been tested in animal models in design work on anti-*Campylobacter* vaccines. These include but are not limited to the *Campylobacter* adhesion-to-fibronectin protein (CadF), DNA binding protein from starved cells (Dps), *C. jejuni* surface-exposed lipoprotein A (JlpA), component protein of the multidrug efflux pump (CmeC), haemolysin co-regulated protein (Hep), flagellin A (FlaA), fibronectin-like protein A (FlpA) and lipoprotein CjaA. The immune response induced by FlaA heat-labile enterotoxin B subunit (FlaA-LTB), CmeC, Hcp, FlpA, CadF and JlpA provided limited protection, that induced by Dps and FlaA did not protect against infection with heterologous strains and the response to CjaA and FlaA was highly variable depending on the dose of the product, route of administration, and application regimen (31). These outcomes suggest that vaccine preparations based on a single antigen offer insufficient protection. Only a multicomponent vaccine has the potential to efficiently diminish *Campylobacter* colonisation of chicken intestines. This was demonstrated by the results obtained by Neal-McKinney (28), who vaccinated chickens with individual CadF, FlaA or FlpA proteins to reduce *C. jejuni* colonisation somewhat, but who achieved the highest level of protection with a trifecta protein containing CadF, FlaA and FlpA. Another example of such an approach is the formulation developed by Vandeputte *et al.* (39), which encompassed six immunodominant antigens: adenosine triphosphate synthase subunit (AtpA), EF-Tu, heat-shock protein 60 (GroEL), trigger factor chaperone (Tig), chemotaxis protein V (CheV) and leucine/isoleucine/valine-binding protein (LivJ). After immunisation of hens, the researchers observed elevated levels of specific IgY against the administered antigens in the egg yolks. Prophylactic feeding of broilers with yolk from vaccinated hens led to a significant reduction in the number of birds colonised by *Campylobacter* (39). Similarly, a protective effect in hens was observed when birds were fed an avirulent strain of *Salmonella* that produced two *Campylobacter* antigens – CadF and FlaA (4). Nonetheless, it is worth considering that the beneficial effect may also have been gained in part through the method of their application.

In the present study, two hybrid proteins – rCjaAEF-Tu and rCjaCD – were prepared. The CjaA protein was frequently used as an antigen, but the results obtained were inconsistent. Some studies reported a reduction in the level of intestinal colonisation by approximately 6 $\log_{10}$, while others showed no effect (3, 42). The second backbone protein, which is CjaC, is highly immunogenic. Studies support the use of CjaA and CjaC lipoproteins as antigens, as they induce strong toll-like receptor 2–mediated innate immune responses in chickens (44). Recent data suggested that the innate immune response may play an important role in protecting chickens from colonisation by *Campylobacter* (32). One of the proteins used as a source of epitopes was EF-Tu, which apart from its main role in polypeptide synthesis has recently been implicated in bacterial pathogenesis (17). The second source of epitopes was the highly immunogenic CjaD lipoprotein (15).

After a series of structural analyses of backbone proteins combined with predicted epitopes, the two hybrid proteins rCjaAEF-Tu and rCjaCD were obtained and purified. It was confirmed that the epitopes selected for their construction were recognised by specific sera, and that they also induced the production of antibodies

which reacted with the native proteins from which the epitopes were derived. The hybrids were then encapsulated in liposomes and orally administered to the chickens. Liposomes are attractive carriers of vaccine antigens. The advantage of liposomes, which are bilayered vesicles composed of amphipathic phospholipids, is that they extend the time of antigen release from their interior and protect their content from excessive proteolysis. In addition, liposomes facilitate uptake by antigen-presenting cells (APCs), leading to stimulation of both humoral and cellular immune responses (34). Furthermore, liposomes can be produced on a large scale, stored for a relatively long time, and most importantly, are considered safe and well tolerated by animals and humans. Some studies have indicated that liposomes can be used successfully to stimulate the immune system in chickens (6, 43).

From our previous experiments, we know that the administration of CjaA and rCjaAD antigens caused an increase in specific IgY and IgA antibodies (14, 25). However, this did not result in any significant reduction of the colonisation of the chicken digestive tract. Therefore, in the planned study, evaluating the protective effect and its outcome was crucial regardless of the immunological status of animals.

Despite the use of hybrid proteins with carefully selected immunogenic epitopes, we did not observe the expected reduction in colonisation in immunised birds. There may be various reasons for this result and further investigation is required. The most probable issues to be considered are liposome–hybrid interaction or antigen selection. Positively charged liposomes were shown to be much more effective than negatively or neutrally charged molecules because they interacted better with negatively charged mucosal surfaces, thus prolonging antigen exposure time (2). They are also more efficiently taken up by APCs (18). However, the results of studies on liposome interactions with APCs, the influence of liposome size, their cargo and the phase state of the bilayer are often contradictory and should be verified on a case-by-case basis. The hybrid proteins we produced were incorporated into the structure of anionic and neutral liposomes, because packing them into cationic forms had failed. A factor deserving investigation may be the charge characteristics of the liposomes. Another aspect requiring investigation is the exact nature of the interaction of the hybrid proteins with liposomes (*e.g.* their surface exposure). Some studies indicate that liposomes elicit a stronger immune response when antigens are exposed on their surfaces rather than encapsulated within. It was shown that packaging was very efficient; however, one of our hybrids, rCjaAEF-Tu, always fragmented during the incorporation process, regardless of the type of liposome used. This observation suggests that the surface presentation of antigens may be a factor driving the efficiency of such vaccine formulations.

Antigen selection is crucial. Both backbone proteins used (CjaA and CjaC) were established to have immunogenic properties in chickens (8, 25, 42). The source of epitopes for the rCjaAEF-Tu hybrid, EF-Tu, was shown to be involved in the pathogenesis of various microorganisms, and importantly it was capable of stimulating secretory IgA in chickens (19). The immunostimulatory effects of CjaD protein, the source of epitopes for the rCjaCD hybrid, were also documented before (15). A similar approach to ours combining various immunogenic determinants was presented by Lou *et al.* (26). A hybrid protein containing epitopes of alkyl hydroperoxide reductase subunit C (AhpC), Omp18 (CjaD) and flagellar L-ring protein precursor (FlgH) was used to immunise specific pathogen-free mice from the BALB/c line, other mice were immunised with only one selected from these three proteins or not immunised at all, and all mice were later challenged with *Campylobacter*. The disease index and protection rate were then calculated based on the disease symptoms observed in the immunised and non-immunised mice. The protection rate was higher for the hybrid protein (80%) than for each of the three proteins separately (30–50%). Although all of our chosen proteins exhibited promising features and both hybrids were positive in Western blots with sera obtained from 7-day-old chickens, it is impossible to predict or guarantee an exact protective effect.

The use of liposomes in multi-antigen vaccines has shown potential, as seen in the vaccines against influenza (Inflexal), hepatitis A (Epaxal) and malaria (Mosquirix). Additionally, incorporating immunostimulatory elements like 5′–C–phosphate–G–3′ (ligands of pattern recognition receptors) into vaccine complexes can modulate the immune response (5).

It is also extremely important to clarify the role of gut microbiota in *Campylobacter* infection in chickens. To date, only a few studies have addressed this issue (41). For example, Kaakoush *et al.* (23) linked the presence of *C. jejuni* in the digestive tract of chickens with a lower abundance of *Lactobacillus* and *Corynebacterium* and a higher abundance of both *Streptococcus* and *Ruminococcaceae*. Changes in the abundance of Clostridiales in response to *Campylobacter* colonisation have also been observed (7, 38). The effects of removing *Campylobacter* from the chicken microbiota are unknown. In the event of its eradication through vaccination, a thorough analysis of the microbiota composition and assessment of the well-being of immunised birds will undoubtedly be necessary.

Conclusion

Although the immunisation described in the present study did not produce the desired results, further exploration of this approach is still worthwhile.

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References

1. Awad W.A., Molnár A., Aschenbach J.R., Ghareeb K., Khayal B., Hess C., Liebhart D., Dublecz K., Hess M.: *Campylobacter* infection in chickens modulates the intestinal epithelial barrier function. Innate Immun 2015, 21, 151–160, doi: 10.1177/1753425914521648.
2. Bernasconi V., Norling K., Bally M., Höök F., Lycke N.Y.: Mucosal Vaccine Development Based on Liposome Technology. J Immunol Res 2016, 2016, 1–16, doi: 10.1155/2016/5482087.
3. Buckley A.M., Wang J., Hudson D.L., Grant A.J., Jones M.A., Maskell D.J., Stevens M.P.: Evaluation of live-attenuated *Salmonella* vaccines expressing *Campylobacter* antigens for control of *C. jejuni* in poultry. Vaccine 2010, 28, 1094–1105, doi: 10.1016/j.vaccine.2009.10.018.
4. Chandran S., Hewawaduge C., Aganja R.P., Lee J.H.: Prokaryotic and eukaryotic dual-expression plasmid-mediated delivery of *Campylobacter jejuni* antigens by live-attenuated *Salmonella*: A strategy for concurrent Th1 and Th2 immune activation and protection in chickens. Dev Comp Immunol 2024, 153, 105134, doi: 10.1016/j.dci.2024.105134.
5. Chatzikleanthous D., Schmidt S.T., Buffi G., Paciello I., Cunliffe R., Carboni F., Romano M.R., O'Hagan D.T., D'Oro U., Woods S., Roberts C.W., Perrie Y., Adamo R.: Design of a novel vaccine nanotechnology-based delivery system comprising CpGODN-protein conjugate anchored to liposomes. J Control Release 2020, 323, 125–137, doi: 10.1016/j.jconrel.2020.04.001.
6. Chiou C.-J., Tseng L.-P., Deng M.-C., Jiang P.-R., Tasi S.-L., Chung T.-W., Huang Y.-Y., Liu D.-Z.: Mucoadhesive liposomes for intranasal immunization with an avian influenza virus vaccine in chickens. Biomaterials 2009, 30, 5862–5868, doi: 10.1016/j.biomaterials.2009.06.046.
7. Connerton P.L., Richards P.J., Lafontaine G.M., O'Kane P.M., Ghaffar N., Cummings N.J., Smith D.L., Fish N.M., Connerton I.F.: The effect of the timing of exposure to *Campylobacter jejuni* on the gut microbiome and inflammatory responses of broiler chickens. Microbiome 2018, 6, 88, doi: 10.1186/s40168-018-0477-5.
8. Cordwell S.J., Len A.C.L., Touma R.G., Scott N.E., Falconer L., Jones D., Connolly A., Crossett B., Djordjevic S.P.: Identification of membrane-associated proteins from *Campylobacter jejuni* strains using complementary proteomics technologies. Proteomics 2008, 8, 122–139, doi: 10.1002/pmic.200700561.
9. European Food Safety Authority and European Centre for Disease Prevention and Control: The European Union One Health 2022 Zoonoses Report. EFSA J 2023, 21, e8442, doi: 10.2903/j.efsa.2023.8442.
10. European Food Safety Authority Panel on Biological Hazards (BIOHAZ), Koutsoumanis K., Allende A., Alvarez-Ordóñez A., Bolton D., Bover-Cid S., Chemaly M., De Cesare A., Hilbert F., Lindqvist R., Nauta M., Peixe L., Ru G., Simmons M., Skandamis P., Suffredini E., Cocconcelli P.S., Fernández-Escámez P.S., Maradona M.P., Querol A., Sijtsma L., Suarez J.E., Sundh I., Vlak J., Barizzone F., Correla S., Herman L.: Update of the list of qualified presumption of safety (QPS) recommended microbiological agents intentionally added to feed or feed as notified to EFSA 17: suitability of taxonomic units notified to EFSA until September 2022. EFSA J 2023, 21, e07746, doi: 10.2903/j.efsa.2023.7746.
11. Finsterer J.: Triggers of Guillain–Barré Syndrome: *Campylobacter jejuni* Predominates. Int J Mol Sci 2022, 23, 14222, doi: 10.3390/ijms232214222.
12. Garvis S.G., Puzon G.J., Konkel M.E.: Molecular characterization of a *Campylobacter jejuni* 29-kilodalton periplasmic binding protein. Infect Immun 1996, 64, 3537–3543, doi: 10.1128/iai.64.9.3537-3543.1996.
13. Gloanec N., Dory D., Quesne S., Béven V., Poezevara T., Keita A., Chemaly M., Guyard-Nicodème M.: Impact of DNA Prime/Protein Boost Vaccination against *Campylobacter jejuni* on Immune Responses and Gut Microbiota in Chickens. Vaccines 2022, 10, 981, doi: 10.3390/vaccines10060981.
14. Godlewska R., Kuczkowski M., Wyszyńska A., Klim J., Derlatka K., Woźniak-Biel A., Jagusztyn-Krynicka E.K.: Evaluation of a protective effect of in ovo delivered *Campylobacter jejuni* OMVs. Appl Microbiol Biotechnol 2016, 100, doi: 10.1007/s00253-016-7699-x.
15. Godlewska R., Wiśniewska K., Pietras Z., Jagusztyn-Krynicka E.K.: Peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria: function, structure, role in pathogenesis and potential application in immunoprophylaxis. FEMS Microbiol Lett 2009, 298, 1–11, doi: 10.1111/j.1574-6968.2009.01659.x.
16. Green M.R., Sambrook J.: *Molecular Cloning, A Laboratory Manual*, Fourth Edition, Cold Spring Harbor Laboratory Press, New York, NY, USA, 2012.
17. Harvey K.L., Jarocki V.M., Charles I.G., Djordjevic S.P.: The Diverse Functional Roles of Elongation Factor Tu (EF-Tu) in Microbial Pathogenesis. Front Microbiol 2019, 10, 2351, doi: 10.3389/fmicb.2019.02351.
18. Henriksen-Lacey M., Christensen D., Bramwell V.W., Lindenstrøm T., Agger E.M., Andersen P., Perrie Y.: Liposomal cationic charge and antigen adsorption are important properties for the efficient deposition of antigen at the injection site and ability of the vaccine to induce a CMI response. J Control Release 2010, 145, 102–108, doi: 10.1016/j.jconrel.2010.03.027.
19. Hermans D., Van Steendam K., Verbrugghe E., Verlinden M., Martel A., Seliwiorstow T., Heyndrickx M., Haesebrouck F., De Zutter L., Deforce D., Pasmans F.: Passive immunization to reduce *Campylobacter jejuni* colonization and transmission in broiler chickens. Vet Res 2014, 45, 27, doi: 10.1186/1297-9716-45-27.
20. Holmes K., Mulholland F., Pearson B.M., Pin C., McNicholl-Kennedy J., Ketley J.M., Wells J.M.: *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology 2005, 151, 243–257, doi: 10.1099/mic.0.27412-0.
21. Jeon B., Saisom T., Sasipreeyajan J., Luangtongkum T.: Live-Attenuated Oral Vaccines to Reduce *Campylobacter* Colonization in Poultry. Vaccines 2022, 10, 685, doi: 10.3390/vaccines10050685.
22. Kaakoush N.O., Castaño-Rodríguez N., Mitchell H.M., Man S.M.: Global Epidemiology of *Campylobacter* Infection. Clin Microbiol Rev 2015, 28, 687–720, doi: 10.1128/CMR.00006-15.
23. Kaakoush N.O., Sodhi N., Chenu J.W., Cox J.M., Riordan S.M., Mitchell H.M.: The interplay between *Campylobacter* and *Helicobacter* species and other gastrointestinal microbiota of commercial broiler chickens. Gut Pathog 2014, 6, 18, doi: 10.1186/1757-4749-6-18.
24. Kobierecka P.A., Olech B., Książek M., Derlatka K., Adamska I., Majewski P.M., Jagusztyn-Krynicka E.K., Wyszyńska A.K.: Cell wall anchoring of the *Campylobacter* antigens to *Lactococcus lactis*. Front Microbiol 2016, 7, 165, doi: 10.3389/fmicb.2016.00165.
25. Kobierecka P.A., Wyszyńska A.K., Gubernator J., Kuczkowski M., Wiśniewski O., Maruszewska M., Wojtania A., Derlatka K.E., Adamska I., Godlewska R., Jagusztyn-Krynicka E.K.: Chicken anti-*Campylobacter* vaccine – comparison of various carriers and routes of immunization. Front Microbiol 2016, 7, 740, doi: 10.3389/fmicb.2016.00740.
26. Lou H., Li X., Sheng X., Fang S., Wan S., Sun A., Chen H.: Development of a Trivalent Construct Omp18/AhpC/FlgH Multi Epitope Peptide Vaccine Against *Campylobacter jejuni*. Front Microbiol 2022, 12, 773697, doi: 10.3389/fmicb.2021.773697.

27. Lübbert C.: Antimicrobial therapy of acute diarrhoea: a clinical review. Expert Rev Anti Infect Ther 2016, 14, 193–206, doi: 10.1586/14787210.2016.1128824.
28. Neal-McKinney J.M., Samuelson D.R., Eucker T.P., Nissen M.S., Crespo R., Konkel M.E.: Reducing *Campylobacter jejuni* Colonization of Poultry via Vaccination. PLoS One 2014, 9, e114254, doi: 10.1371/journal.pone.0114254.
29. Pawelec D.P., Korsak D., Wyszyńska A.K., Rożynek E., Popowski J., Jagusztyn-Krynicka E.K.: Genetic diversity of the *Campylobacter* genes coding immunodominant proteins. FEMS Microbiol Lett 2000, 185, 43–49, doi: 10.1111/j.1574-6968.2000.tb09038.x.
30. Poly F., Noll A.J., Riddle M.S., Porter C.K.: Update on Campylobacter vaccine development. Hum Vaccin Immunother 2019, 15, 1389–1400, doi: 10.1080/21645515.2018.1528410.
31. Pumtang-on P., Mahony T.J., Hill R.A., Vanniasinkam T.: A Systematic Review of *Campylobacter jejuni* Vaccine Candidates for Chickens. Microorganisms 2021, 9, 397, doi: 10.3390/microorganisms9020397.
32. Reid W.D.K., Close A.J., Humphrey S., Chaloner G., Lacharme-Lora L., Rothwell L., Kaiser P., Williams N.J., Humphrey T.J., Wigley P., Rushton S.P.: Cytokine responses in birds challenged with the human food-borne pathogen *Campylobacter jejuni* implies a Th17 response. R Soc Open Sci 2016, 3, 150541, doi: 10.1098/rsos.150541.
33. Sahin O., Luo N., Huang S., Zhang Q.: Effect of *Campylobacter*-Specific Maternal Antibodies on *Campylobacter jejuni* Colonization in Young Chickens. Appl Environ Microbiol 2003, 69, 5372–5379, doi: 10.1128/AEM.69.9.5372-5379.2003.
34. Schwendener R.A.: Liposomes as vaccine delivery systems: a review of the recent advances. Ther Adv Vaccines 2014, 2, 159–182, doi: 10.1177/2051013614541440.
35. Shoaf-Sweeney K.D., Larson C.L., Tang X., Konkel M.E.: Identification of *Campylobacter jejuni* Proteins Recognized by Maternal Antibodies of Chickens. Appl Environ Microbiol 2008, 74, 6867–6875, doi: 10.1128/AEM.01097-08.
36. Silva J., Leite D., Fernandes M., Mena C., Gibbs P.A., Teixeira P.: *Campylobacter* spp. as a foodborne pathogen: a review. Front Microbiol 2011, 2, 200, doi: 10.3389/fmicb.2011.00200.
37. Studier F.W.: Protein production by auto-induction in high-density shaking cultures. Protein Expr Purif 2005, 41, 207–234, doi: 10.1016/j.pep.2005.01.016.
38. Thibodeau A., Fravalo P., Yergeau É., Arsenault J., Lahaye L., Letellier A.: Chicken Caecal Microbiome Modifications Induced by *Campylobacter jejuni* Colonization and by a Non-Antibiotic Feed Additive. PLoS One 2015, 10, e0131978, doi: 10.1371/journal.pone.0131978.
39. Vandeputte J., Martel A., Canessa S., Van Rysselberghe N., De Zutter L., Heyndrickx M., Haesebrouck F., Pasmans F., Garmyn A.: Reducing *Campylobacter jejuni* colonization in broiler chickens by in-feed supplementation with hyperimmune egg yolk antibodies. Sci Rep 2019, 9, 8931, doi: 10.1038/s41598-019-45380-z.
40. Vorwerk H., Mohr J., Huber C., Wensel O., Schmidt-Hohagen K., Gripp E., Josenhans C., Schomburg D., Eisenreich W., Hofreuter D.: Utilization of host-derived cysteine-containing peptides overcomes the restricted sulphur metabolism of *Campylobacter jejuni*. Mol Microbiol 2014, 93, 1224–1245, doi: 10.1111/mmi.12732.
41. Wyszyńska A.K., Godlewska R.: Lactic Acid Bacteria – A Promising Tool for Controlling Chicken *Campylobacter* Infection. Front Microbiol 2021, 12, 703441, doi: 10.3389/fmicb.2021.703441.
42. Wyszyńska A.K., Raczko A., Lis M., Jagusztyn-Krynicka E.K.: Oral immunization of chickens with avirulent *Salmonella* vaccine strain carrying *C. jejuni* 72Dz/92 cjaA gene elicits specific humoral immune response associated with protection against challenge with wild-type *Campylobacter*. Vaccine 2004, 22, 1379–1389, doi: 10.1016/j.vaccine.2003.11.001.
43. Yaguchi K., Ohgitani T., Noro T., Kaneshige T., Shimizu Y.: Vaccination of Chickens with Liposomal Inactivated Avian Pathogenic *Escherichia coli* (APEC) Vaccine by Eye Drop or Coarse Spray Administration. Avian Dis 2009, 53, 245–249, doi: 10.1637/8475-092908-Reg.1.
44. de Zoete M.R., Keestra A.M., Roszczenko P., van Putten J.P.M.: Activation of Human and Chicken Toll-Like Receptors by *Campylobacter* spp. Infect Immun 2010, 78, 1229–38. doi: 10.1128/IAI.00897-09.