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Vaccination of chickens against *Campylobacter*

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Abstract

The Gram-negative bacterium *Campylobacter* is the leading cause of bacterial entero-colitis in humans and is associated with the occurrence of life-threatening auto-immune based neurological disorders. Chickens, which are often heavily colonized with *Campylobacter* without signs of pathology, are considered the most important source for human infection. Although vaccination is a well established and effective method to combat various microbes in poultry, a commercial vaccine against *Campylobacter* has not yet been developed. For the development of such a vaccine, three main challenges can be identified: (1) the identification of novel cross-protection-inducing antigens, (2) the induction of a rapid, potent immune response, and (3) the development of novel adjuvants to further stimulate immunity against *Campylobacter*. The rapidly emerging knowledge of the biology of *Campylobacter* in combination with the recent advances in the fields of molecular vaccinology and immunology provide the required setting for the development of an effective vaccine against *Campylobacter* in poultry.

Keywords: *Campylobacter*; Vaccine; Chicken

1. Introduction

Each year, the Gram-negative bacterium *Campylobacter* is responsible for an estimated 400 million human cases of entero-colitis worldwide, making it the leading cause of bacterial foodborne disease and a major causative agent of traveller's disease [1–3]. In a limited number of cases the enteric manifestations are followed by sequelae, such as reactive arthritis and the life-threatening neuropathy Guillain-Barré Syndrome (GBS) [1,4,5]. Estimated incidences of human campylobacteriosis in industrialized countries vary from 21.9/100,000 (US) to 396/100,000 (New Zealand). In developing countries, approximately 40–60% of young children is estimated to become infected every year and high numbers of asymptomatic carriage are reported [6–8]. Altogether, *Campylobacter* species affect the health of millions of people worldwide with an estimated annual economical burden of up to 8 billion dollars in the US alone [9].

At present, 17 *Campylobacter* species have been identified that can be separated into more than 60 Penner serotypes (heat-stable antigens) and more than 100 Lior serotypes (heat-labile antigens). Two thermophilic species, *C. jejuni* ssp. *jejuni* and *C. coli* (further referred to as *C. jejuni* and *C. coli*, or together as *Campylobacter*) are responsible for the vast majority of human campylobacteriosis (∼90% and ∼10%, respectively). Although *C. jejuni* and *C. coli* are frequently isolated from the digestive tract of a wide variety of warm-blooded animals, (broiler) chickens are considered as the most important source of human infection [10,11]: as much as 70% of raw poultry meat products sold in the US in 1999/2000 was found to be contaminated with high levels of viable *Campylobacter* [12].

Several strategies have been applied to reduce *Campylobacter* counts on chicken meat, including attempts to eliminate *Campylobacter* from the farms by increasing biosecurity and the separation of contaminated flocks, and by

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improving hygiene during the process of slaughtering. In addition, several experimental approaches like the reduction of colonization by competitive exclusion, antibacterial agents, or phage therapy are being investigated for their efficacy [13–15]. Although these measures undoubtedly will help to control shedding of *Campylobacter* by the animals and may reduce the number of positive flocks, vaccination of poultry against *Campylobacter* will probably be most effective and remains a major goal.

This review discusses the current status of vaccine development against *Campylobacter* in poultry. Major hurdles and focus points for the development of an effective vaccine are identified by comparing the course of infection and the seemingly effective immune response against *Campylobacter* in humans with the colonization and immune response in chickens.

2. *Campylobacter* in humans

2.1. Infection of the digestive tract

Campylobacter infection in humans is initiated by ingesting as little as 500 bacteria that, aided by their corkscrew-shape and high motility, move easily through the intestinal tract and colonize preferably the distal ileum and colon [16–19]. Here, the bacterium resides specifically in the mucosal layer, disrupts the epithelial barrier, and initiates an inflammatory response. This may give rise to clinical symptoms that range from mild watery to voluminous and bloody diarrhoea accompanied by headache, abdominal pain, fever, malaise and occasional vomiting [20]. The molecular events that lead to acute intestinal inflammation largely remain to be defined. In recent years, a series of virulence determinants (e.g. Peb1, JplA, CadF and FlaC) that influence bacterial adhesion or invasion *in vitro* have been identified [16,21,22]. Furthermore, the presence of toxins in certain strains of *Campylobacter* has been suggested to enhance the inflammation [23]. The contribution of each of these factors to the development of entero-colitis however, remains to be established.

2.2. Immune response in humans

Campylobacter infections are generally self-limiting; diarrhoea usually lasts no more than 3 to 5 days, while other symptoms gradually resolve the following week [20]. From the second week after the infection, *Campylobacter*-specific antibodies against numerous antigens including flagellin, major outer membrane protein (MOMP) and lipopolysaccharides (LPS), can be detected in the serum and mucosal secretions [24–26]. Levels of serum IgG and IgM show a strong peak around 8 to 14 days post-infection and then gradually drop over a period of about 2 months (IgM) to 1 year (IgG). Serum IgA levels also peak at 1–2 weeks of infection but return to pre-infection levels within several weeks [27–29]. In the intestine, specific IgA increases within a week and drops rapidly after 16 to 20 days of infection [30].

The generation of anti-*Campylobacter* antibodies is likely beneficial for clearance of the bacterium. In addition, several studies suggest that the presence of specific antibodies may provide (some) protection against clinical disease upon re-infection. In developing countries, where people are continuously challenged with *Campylobacter* and often colonized without clinical symptoms, the occurrence of asymptomatic carriership strongly correlates with increased antibody titers [31]. Furthermore, in human volunteer studies re-challenge with the homologous *Campylobacter* strain has been shown to result in a less severe clinical outcome [19]. Finally, breast milk with maternal IgA directed against several *Campylobacter* surface antigens has been shown to protect young children against *Campylobacter*-induced diarrhoea [32–34].

3. *Campylobacter* in chickens

3.1. Colonization of broiler chickens

Chicken are also easily colonized with *Campylobacter*, although considerable variation between bacterial strains and the specific breed and age of the broiler have been reported [35–38]. Chickens usually encounter *Campylobacter* at a young age and faecal shedding results in a rapid spread throughout the entire flock. Depending on the geographical region and season, the percentage of *Campylobacter*-positive flocks in Europe reaches up to 90% [39]. The two dominant *Campylobacter* species that colonize chickens are similar to those in humans, although the proportion of *C. jejuni* and *C. coli* is different (∼65% and ∼35% in broilers, respectively) (Jacobs-Reitsma WF, personal communication). Thus far, molecular typing has not revealed intrinsic differences between chicken and human isolates. However, these studies are complex as most *Campylobacter* strains do not have a stable genotype due to a high frequency of DNA exchange between strains, and chickens (but not humans) are often colonized with multiple strains [40,41].

Once colonized, *Campylobacter* does not elicit the potent inflammatory response that can be observed in humans but rather seems to behave as a commensal bacterium of the chicken gut. The bacterium colonizes the caeca, large intestine and cloaca of chicken in numbers up to 10^9 per gram of faeces [42,43] without apparent signs of pathology. They are found in the lumen and the mucus, and penetrate deep into the intestinal crypts in close proximity to the epithelium, without cellular adherence or invasion. In experimental infections, the rapid peak in intestinal bacterial numbers is often followed by a slow decrease from week 4 up to the point of slaughtering at 6 weeks of age, with some birds able to completely clear the bacteria [44]. Some studies report the presence of bacteria in the spleen, liver and blood in young chicks, suggesting that, shortly after hatching, *Campylobacter* may gain access to the

deeper tissues [45,46]. Whether this is caused by insufficient maturation of the mucosal tissue, the absence of endogenous bacterial flora, and/or immune factors, is unknown.

The molecular basis for the apparent different lifestyle of *Campylobacter* in the chicken and human host remains to be defined. However, it has been demonstrated that effective colonization of the chicken gut require several bacterial proteins including flagellin, CadF and CiaB, as well as a functional protein N-glycosylation machinery and intact two-component signal transduction systems, such as RacR/RacS, FlgS/FlgR and DccR/DccS. This suggest that these bacterial factors may be potential vaccine targets [47–51].

3.2. Immune response in chickens

Several studies have shown a strong correlation between increasing levels of *C. jejuni*-specific antibodies in chicken and the reduction of bacterial shedding observed with duration of the colonization, suggesting the development of an effective response [52–54]. In general, *Campylobacter*-specific serum IgG, IgA and IgM levels rise gradually starting at week 2–3 after experimental inoculation, while biliary and/or intestinal IgA are present starting from week 3 to 4. Flagellin is generally the first antigen to be recognized by all antibody isotypes. During the following 8 weeks, antibodies directed against numerous other bacterial antigens including the major outer membrane protein, are induced [53,54]. Overall, the main difference in host immune response towards *Campylobacter* between humans and chickens seems to be within the first week of infection, when the intestinal tracts of both species are heavily colonized. In humans but not in chicken, this is accompanied with an inflammatory response and tissue damage. The absence of a strong activation of the innate immune defense in the chicken may be an important reason for the slow and moderate antibody response against *Campylobacter* in this species.

3.3. Maternal antibodies

Young chicks have considerable levels of *Campylobacter*-specific maternal IgG at hatch. These antibody levels decrease gradually during the first 3 weeks of post-hatching [52,55]. Maternal antibodies are transferred from infected layer hens to the oocyte during embryonic development and serve to protect against pathogens in the weeks post-hatch when the immune system is still developing. The observation that chicks in flocks are often free of *Campylobacter* during the first week post-hatch suggests a role for these antibodies in protection against colonization. Experimental data show that maternal IgG from 1-day-old chicks recognizes *C. jejuni* membrane proteins in the wide range of 19–107 kDa and exhibit bactericidal activity [55]. In addition, 3-day-old chicks from *C. jejuni*-infected hens exhibit a 2–4 day delay in colonization when compared to chicks from non-infected hens, by both a homologous and heterologous strain [56]. These observations suggest that maternal antibodies may offer some level of protection against colonization by *Campylobacter*, although it cannot be excluded that additional factors, like the intestinal flora or the maturation of the intestinal tract contribute to the delayed colonization.

4. Studies on vaccination against *Campylobacter* in chickens

4.1. Goal of vaccination

A successful broiler chicken vaccine should meet the following standards: (1) a protective response has to be induced quickly as young chicks come into contact with *Campylobacter* very early on in life, (2) immunity should be cross-protective for both *C. jejuni* and *C. coli*, together comprising a great number of different serotypes, (3) the vaccine should be cost-effective and easy to deliver as massive numbers of chicken have to be immunized, and (4) the vaccine must be safe for animals and humans and not leave residues. Overall, vaccination should prevent colonization or cause a strong (more than 2–3 log) reduction of bacterial numbers in colonized animals. Currently, such a *Campylobacter* vaccine does not exist but, as outlined below and in Table 1, several vaccine development strategies are currently being employed to achieve this goal.

4.2. Protective role of Campylobacter-specific antibodies

Proof-of-principle that *Campylobacter*-specific antibodies can effectively reduce *Campylobacter* colonization of chickens was obtained when *C. jejuni* was preincubated with purified *Campylobacter*-specific immunoglobulins purified from the bile of colonized broiler chickens. This significantly increased the dose needed for colonization (CD_{50}) by 50% [57]. The protective properties of *Campylobacter* antibodies were further assessed by Cawthraw et al. [58] who cleared *C. jejuni* from experimentally colonized chickens and re-challenged them with the homologous *C. jejuni* strain. After a 4-week colonization period and 10 days of erythromycin treatment, re-challenge of the birds with 4×10^5 cfu at week 7 or 8 resulted in mean caecal colonization numbers that were ~1 log lower than the control group. When these birds were challenged with a dose 100 times higher than the controls, colonization levels remained lower.

A similar approach of using viable bacteria to generate a protective immune response involved the oral inoculation of 5-day-old chickens with *C. jejuni* strains attenuated in colonization due to disrupted *cadF*, *pldA*, *dnaJ* or *ciaB* genes. Subsequent challenge of the animals with the parent strain however, resulted in unaltered colonization levels compared to naive animals [51]. In this study, the presence of viable *C. jejuni* in the chicken's intestine may have been too short to generate sufficient protective immunity, as measurement of the immune response after experimental colonization of

Table 1
Overview of studies on vaccination against *Campylobacter* in chickens

Type of vaccine	Administration	Effect	Ref.
Experimental colonization with wildtype *C. jejuni*	Orally	∼1 log reduction upon homologous challenge	[58]
Experimental colonization with non-colonizing mutant of *C. jejuni*	Orally	No effect upon homologous challenge	[51]
Formalin-inactivated *C. jejuni* +/− LT	Orally with boosters	∼1.5 log reduction upon homologous challenge no additional effect of LT	[53]
Formalin-inactivated *C. jejuni* +/− LT or CT	Orally with boosters	No effect upon homologous challenge	[58]
Formalin-inactivated *C. jejuni* complete Freund's adjuvant	SC with booster	Some reduced shedding during first 2 weeks upon homologous challenge only	[61]
Heat-killed *C. jejuni*	*In ovo* with oral booster	Generation of flagellin-specific serum IgG, IgM and IgA, and IgA in bile and intestine	[62]
Native flagellin +/− heat-killed *C. jejuni*	IP with IP or oral booster	∼1–2 log reduction upon homologous challenge	[66,67]
Recombinant flagellin fused to LT	Orally with booster	Reduction of *C. jejuni* positive chickens (40/145 vs. 70/142 in control group)	[68]
Plasmid DNA containing the flagellin gene	IM with booster	2 log reduction upon homologous challenge	[69]
67, 73.5 and 77.5 kDa immunogenic *C. jejuni* proteins	IP	No effect upon homologous challenge	[66]
Attenuated *Salmonella* expressing CjaA	Orally	>6 logs reduction upon homologous challenge	[81]

chicken indicates that *Campylobacter*-specific antibodies can first be detected after several weeks of infection [52,54].

Together, the limited available data suggest that *Campylobacter*-specific antibodies generated during natural and experimental colonization provide some protection against renewed colonization and thus that the induction of a protective response by vaccination may be feasible. However, the studies also indicate that a much stronger immune response has to be generated than is observed during natural colonization of chickens with *Campylobacter*.

4.3. Killed whole-cell vaccines

Killed whole-cell vaccines (WCV) are relatively safe, cost-effective, easy to produce, and have been demonstrated to prevent disease caused by intestinal pathogens [59,60]. In chickens, several WCV have been tested as vaccines against *Campylobacter*. Formalin-inactivated *C. jejuni* (10^9 cfu) have been administered orally to broilers at multiple time points within 16 days post-hatch, with and without *Escherichia coli* heat-labile toxin (LT) [53]. After challenge (by seeder animals) with the homologous strain the numbers of *C. jejuni* isolated from the caecum were lower in all vaccinated groups compared to the control groups, with a maximum reduction of ∼1.5 log in one group (1.0×10^8 cfu/g versus 1.4×10^9 cfu/g). Bile IgA levels were comparable between vaccinated and non-vaccinated animals, but IgA from vaccinated animals recognized more *C. jejuni* antigens than control IgA, especially in the range of antigens with molecular masses between 14 and 45 kDa. Unexpectedly, the addition of LT did not enhance the immune response.

Two other vaccination studies yielded more disappointing results. In one study oral vaccination with 5×10^7 formalin killed *C. jejuni* on days 3, 10 and 17 did not cause a reduction of caecal colonization after challenge with the homologous strain at week 5, even in the presence of LT or cholera toxin (CT) [58]. No antibodies against *C. jejuni* were detected in the sera or intestinal scrapings, although several birds did develop LT- or CT-specific antibodies. In the second study, *C. jejuni*-specific antibodies in serum were generated in chickens injected subcutaneously with 10^{10} formalin-inactivated bacteria with complete Freund's adjuvant, particularly in chicken 4 or 7 weeks of age [61]. The antibody titers were two-fold higher than those generated during oral infection. However, colonization of the homologous strains was only slightly influenced during the first 2 weeks, showing either a reduction or a retarded peak of *C. jejuni* faecal excretion. Colonization levels of a heterologous strain were not affected.

In an interesting study by Noor et al. [62], *in ovo* vaccination of chick embryos with 10^8 heat-killed *C. jejuni* resulted in flagellin-specific IgG, IgM and IgA in serum, and IgA in bile and intestinal scrapings. After an oral booster at day 7 post-hatch, serum antibody levels tended to be lower than before the boost, while secreted IgA levels were strikingly higher. This was reflected by high numbers of IgM and IgA-containing cells in the duodenum and ileum, respectively. No subsequent challenges of vaccinated chicks were performed, leaving unanswered the level of protection provided by the mucosal antibodies induced in this study.

The seemingly limited success of killed WCV may have several reasons. One factor may be that the bacteria used in WCV were always grown in standard culture medium, which might not result in expression of surface structures necessary for colonization of the chicken's intestine. Furthermore, the addition of adjuvants to enhance a mucosal immunity did not sufficiently increase an intestinal response, suggesting that mammalian adjuvants might not have the desired effect

in avian species, although cholera toxin has been reported to be immunostimulatory in chickens [63,64]. Finally, the killed *C. jejuni* themselves may not offer strong immunostimulatory properties or even possess immune-suppressive activities that limit the development of an effective immune response.

4.4. Flagellin-based vaccines

The immunodominant antigen of *Campylobacter* in the majority of the studies described above is the flagellin protein, the major subunit of the bacterial flagellum. *Campylobacter* flagellin is crucial for efficient colonization of the chicken gut and therefore may serve as a protective antigen [54,65]. Several studies have explored the effectiveness of flagellin subunit vaccines, with variable success. In two studies, Widders et al. [66,67] immunized chickens with native flagellin alone or in combination with heat-killed bacteria by intraperitoneal (IP) injection followed by an IP or oral booster. Serum IgG and IgM, and intestinal IgG antibody titers were highest when chicks were vaccinated with the flagellin/whole-cell combination administered IP/IP. This resulted in a ~1–2 log reduction of caecal colonization after challenge.

Oral vaccination with 1 mg of recombinant flagellin fused to heat-labile toxin of *E. coli* showed similar results [68]. After two immunizations at weeks 2 and 4 post-hatching, and challenge with 2×10^8 *C. jejuni* at week 3, the percentage of *C. jejuni*-positive chickens at week 5 (housed in groups of 10) was reduced from 49.3% (70/142) in the non-vaccinated group to 27.6% (40/145) in the vaccinated animals. An alternative approach has been the vaccination by intramuscular (IM) administration of plasmid DNA containing the *C. jejuni* 11168 *flaA* gene at days 2 and 18 of age. This resulted in a 2 log reduction of mean caecal colonization when chicken were challenged with the homologous strain. However, no specific antibodies could be detected [69].

Overall, these results indicate that vaccination with flagellin-based vaccines can induce an immune response that influence colonization levels in the gut. However, in order to be used in an effective vaccine, higher levels of protection are desired. As with the WCV, adjuvants, when used, did not seem to effectively boost a strong intestinal immune response. Also, the effectiveness of the generated flagellin-specific antibodies should be further examined, for several reasons. First, the majority of antibodies seem to recognize epitopes on the flagellin that are not surface exposed in the flagellar structure [66]. Second, a substantial part of the flagellin-specific antibodies may recognize phase-variable glycosylation residues on the surface exposed regions of flagellin [70]. *Campylobacter* is able to vary both the amount and the type of these pseudaminic acid residues and thereby evade the recognition of flagellin-specific antibodies [71,72]. Finally, the surface exposed region of flagellin, like those of many other bacteria, exhibits large variation between *Campylobacter* strains, which may clearly limit the level of cross-protection of flagellin-vaccines.

4.5. Other types of vaccines

Besides flagellin, several other proteins are immunogenic during *Campylobacter* colonization of humans, mice, and chickens. During human infection, high levels of serum antibodies are generated against several proteins, including Peb1 (28 kD), Peb3 (30 kD) and Omp18 [73,74]. Peb1 has been shown to function both as a surface exposed adhesin to mammalian cells and as an aspartate/glutamate-binding protein of an ABC transporter, making this protein a potential candidate vaccine antigen [75,76]. However, to our knowledge, none of these antigens has thus far been included in a chicken vaccination trials. Only Peb1, expressed in an attenuated *Salmonella* strain, was examined for the induction of protective immunity in mice, but failed to reduce intestinal colonization when challenged with *C. jejuni* [77]. Peb1 is now thought to be localized mainly in the periplasm.

In chickens, a series of unidentified *C. jejuni* proteins are recognized by antisera after immunization with a combination of killed whole *Campylobacter* and flagellin, including proteins of with molecular masses of 67, 73.5 and 77.5 kDa. When included in an intraperitoneal vaccine, these three antigens did not significantly reduce caecal *C. jejuni* levels [66].

Recently, two putative ABC transporter proteins (CjaA and CjaC) and a putative petidoglycan-associated lipoprotein (CjaD) have been identified as important immunogens using a *C. coli* specific rabbit antiserum [78,79]. For CjaA and CjaC, western-blot analysis revealed antigenic conservation among all 30 different serotypes of both *C. jejuni* and *C. coli* tested [80]. Oral immunization of chickens with an attenuated *Salmonella* strain expressing *C. coli* CjaA (at day 1 post-hatch and boosted at week 2) generated *C. coli* membrane proteins-specific IgG in serum and IgA in intestinal fluid, and reduced colonization by at least 6 logs in the majority of birds challenged with *C. jejuni* at 4 weeks of age [81]. These remarkable results clearly show the potential of the CjaA protein in combination with the attenuated *Salmonella* as an inter-species cross-protective vaccine. Whether protection is obtained by blocking of the transport function of the CjaA protein and/or by antibody-induced bacterial aggregation and whether similar type of transporters may be suitable vaccine antigens as well, remains to be investigated. Future experiments should also include a control group containing chickens vaccinated with *Salmonella* carrying the vector plasmid without CjaA to determine the precise role of the attenuated *Salmonella* strain in the development of an effective intestinal response.

4.6. Campylobacter vaccines in animal models

Several studies investigated the possibilities of vaccination against *C. jejuni* colonization in mice, ferrets, rabbits or non-human primates. Although these animals are generally used as models for human pathogenesis and immunity, and do not simulate intestinal colonization as seen in chickens, interesting observations have been made that may aid vaccine development in poultry.

In general, experimental infection of mice, rabbits, ferrets and non-human primates with *Campylobacter* induced significant protective immunity against re-challenge as demonstrated by a reduction or even absence of intestinal *Campylobacter* and clinical symptoms [82–87]. Several different killed WCV also provided immunity against re-challenge; in mice and rabbits repeated oral immunization with 10^9 killed bacteria reduced colonization when challenged with the homologous (but not with a heterologous) strain. This effect required the presence of LT as adjuvant [83]. Even more striking was the effect of vaccination of ferrets with a WCV without additional adjuvants. This yielded protection against both the homologous and a heterologous *C. jejuni* strain [88].

The basis for the clear differences in vaccine efficiency between chickens and mammals is unknown. It can be speculated that the induction of intestinal inflammation in the mammalian which appears absent in the avian species results in a more effective immune response. Furthermore, killed WCV may exhibit species-specific immunostimulatory properties as seems to be the case for mucosal adjuvants, such as LT.

5. Novel vaccine development strategies

5.1. Identification of novel candidate antigens

The genomic and phenotypic instability of most Campylobacters, caused by frequent DNA rearrangements, DNA transfer among strains as well as the presence of a large number of phase-variable genes, has created a large diversity of *Campylobacter* strains. One of the major challenges in the development of a vaccine against *Campylobacter* is the identification of protective antigens that are conserved among all serotypes of *C. jejuni* and *C. coli*. Surface exposed antigens generally have the highest potential as vaccine candidates, although factors that *Campylobacter* secretes into the environment and that are important for colonization may also provide protection. Recent advantages in the fields of immunology and genomics have provided exciting opportunities, which can be particularly useful in the identification of novel *Campylobacter* candidate antigens, as shown in a recent paper by Prokhorova et al. [89], where over 110 *C. jejuni* surface proteins were identified using mass spectrometry analysis. Another example is the use of *C. jejuni* genomic DNA expression libraries, as was successfully demonstrated by Pawelec et al. [78]. In their approach, a great number of *C. jejuni* proteins were screened with serum from hyper-immune rabbits to identify new antigens, especially those that are not expressed by bacteria grown in standard culture medium. This method could be further exploited by screening serum from chickens to evaluate species specificity of immunogenic proteins.

Examination of *in vivo* gene expression, for instance by using whole genome microarrays and RT-PCR, presents a more fundamental approach for the improvement of *Campylobacter* vaccines and the identification of protective antigens. In addition, a thorough understanding of environmental signals and regulatory systems controlling *Campylobacter* (surface) proteins or structures essential during colonization can greatly improve the quality of killed whole-cell vaccines. An example of this is the recent illustration of increased *C. jejuni* bacterial adhesion and invasion of eukaryotic cells in the presence of bile salts [90].

The most recent advance that may facilitate vaccine development is the increasing number of available complete *Campylobacter* genome sequences. This provides novel opportunities for reverse genetic approaches to identify putative candidate vaccine antigens that are conserved among strains.

5.2. Boosting of the chicken immune system

Effective vaccination requires targeted manipulation of both the innate and the adaptive immune system. In mammals, the significance of the innate immune response as a key regulatory element of the adaptive immune systems has become particularly evident with the discovery of the family of Toll-like receptors (TLRs). These membrane receptor proteins are capable of sensing the presence of pathogens by their specific molecular patterns, e.g. lipoproteins (TLR2), LPS (TLR4), flagellin (TLR5) or unmethylated CpG DNA (TLR9) and subsequently direct the immune system towards the appropriate response [91]. Activation of the 'right' panel of TLRs is likely an important element in the development of immunity against *Campylobacter*. At this time, our knowledge of the interaction of *Campylobacter* with TLRs is still limited. It has been demonstrated that *C. jejuni* DNA is able to stimulate human TLR9, but to a lesser extent than DNA of other bacteria, such as *Salmonella* and *E. coli*, probably because of differences in GC content [92]. Furthermore, *Campylobacter* belongs to the group of mucus-based ε-proteobacteria, that also include *Helicobacter pylori* and *Wolinella succinogenes*, that have evolved a flagellin that does not activate human TLR5 [93,94]. The significance of these immuno-evasion-like properties of *Campylobacter* for the development of an adequate immune response is unknown, but should be considered in vaccine development.

Many homologues of the human TLRs have been identified in chickens, but functional studies are scarce, especially in combination with *Campylobacter*-derived TLR ligands [95–102]. It cannot be excluded that species differences in TLR-mediated innate responses contribute to the differences in pathology between mammals and chickens. Furthermore, as LPS is a potent mucosal adjuvant and part of both live attenuated and killed WCVs, it seems important to investigate how chickens respond to *Campylobacter* LPS. It has been noted that a high dose of *E. coli* LPS can activate cells via chicken TLR2, which has led to the suggestion that chickens may have a more elaborate system for sensing and responding to LPS than mammals [95].

Since boosting of the immune system seems to be indispensable for the development of an adequate immune response against *Campylobacter* antigens and a number of classical adjuvants do not seem to have the desired effect during vaccination studies in chickens, thorough evaluation of adjuvant activity in chickens may also aid vaccine development. The powerful mammalian adjuvants monophosphoryl lipid A and CpG, both acting on TLRs as described above, have not yet been specifically included in *Campylobacter* vaccines and are not yet well-defined as avian mucosal adjuvants. Worth mentioning is the growing number of chicken cytokines that were shown to possess useful immunostimulatory properties. Although relatively expensive, chicken cytokines may present a promising alternative to classical adjuvants [103,104].

5.3. Vaccine delivery

Vaccine delivery is an important aspect of vaccination in particular with respect to the time point(s) of immunization, the way of administration, and the vaccine vehicle. To determine the optimal time points for vaccination, a good understanding of the development of the immune system is required. This is especially crucial for *Campylobacter* vaccination, as chicks have to be immunized and protected at a very young age. Functional macrophages are found early in embryonic development in the liver (embryonic day (ED) 12) or in the spleen (ED16) [105]. However, the chicken adaptive immune system further develops during the first 2 weeks after hatching and continues to mature up to slaughtering at 6 weeks. The gut-associated lymphoid tissue (GALT) develops in two waves. During the first week of life, increasing numbers of T cells and natural killer cells colonize the entire intestine, while in the second week post-hatching, another wave of B and T cells colonization and maturation occurs probably largely driven by the colonizing gut flora. At hatch, the caecal tonsils are undeveloped and contain mainly T cells, but house increasing numbers of B cells during further development over a period of 6 weeks [106,107].

Clearly, the immaturity of the chicken immune system in the first 2 weeks post-hatching may have its repercussions on early vaccination as evidenced by the relative poor antibody responses in chicks immunized before the age of 10 days [106,108]. Development of strategies to boost maturation of the immune system or, alternatively, strengthening of maternal immunity, may provide solutions to this problem.

Besides generating an effective immune response, a vaccine against *Campylobacter* in poultry needs to be cost-effective. For a chicken vaccine, two types of vaccine delivery, namely oral and *in ovo*, are particularly suitable. Oral immunization, used with several commercial vaccines, is the classical method for obtaining mucosal immunity and can be efficiently applied via drinking water or by aerosol application. *In ovo* vaccination is a relatively new and fully automatic method to vaccinate huge numbers of eggs, typically 20,000 to 30,000 per hour, and is successfully applied for vaccination against viral infections, such as Marek's disease, infectious bursal disease and Newcastle disease [109]. Eggs are injected through the egg shell into the amnion or embryo at day 18 of incubation, only a few days prior to hatching. The generally strong immune response after *in ovo* immunization seems contradictory to the immaturity of the chick's immune system. However, it has been noticed that *in ovo* vaccination may stimulate both the innate as the adaptive immune system more efficiently than post-hatch vaccination. In addition, immunization at the embryonic phase may provide chicks with a 'head start' against challenging pathogens compared to birds vaccinated at later timepoints [110]. Routine *in ovo* vaccination against bacterial infection has not been developed but may be worthwhile exploring as it provides an efficient and potentially effective method for vaccine delivery.

Box 1: Major research questions for vaccine development against *Campylobacter*

1. Which novel, surface exposed *Campylobacter* antigens are expressed during colonization of the chicken's intestinal tract?
2. Do potential vaccine antigens exhibit a broad cross-protection for *Campylobacter* serotypes?
3. Does the chicken's innate immune system have a similar role in directing the adaptive immune response as found for mammals?
4. Which TLR ligands are present in *Campylobacter* and to what extend do they influence immunity in chickens?
5. Which mucosal adjuvants should be used during vaccination of chickens?
6. What is the optimal and earliest timepoint for vaccination?
7. Is *in ovo* vaccination applicable to vaccination against *Campylobacter*?

6. Closing remarks

The positive results obtained with experimental vaccines in several mammalian species suggest that successful vaccination is feasible. In poultry, the apparent protection against *Campylobacter* provided by maternal immunity and the (limited) protection against re-infection indicate that also in this species vaccination could serve as a potential strategy to fight *Campylobacter*. The rapid advances in genomics, proteomics, reverse vaccinology and targeted modulation of the (innate) immune response, hold promise that the major issues that still need to be addressed to develop an effective vaccine (summarized in Box 1) can be resolved in the near future.

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