Research Note: Lyophilization of hyperimmune egg yolk: effect on antibody titer and protection of broilers against *Campylobacter* colonization

Jasmien Vandeputte,[*,1] An Martel,[*] Gunther Antonissen,[*,†] Marc Verlinden,[*] Lieven De Zutter,[‡] Marc Heyndrickx,[*,§] Freddy Haesebrouck,[*] Frank Pasmans,[*] and An Garmyn[*,1]

[*]*Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, B9820 Merelbeke, Belgium;* [†]*Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University, B9820 Merelbeke, Belgium;* [‡]*Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, B9820 Merelbeke, Belgium; and* [§]*Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Technology & Food Sciences Unit, B9090 Melle, Belgium*

ABSTRACT Oral administration of antibodies is a promising strategy against various infectious diseases. Previously, it was demonstrated that passive immunization by providing hyperimmune egg yolk through the feed reduces *Campylobacter jejuni* colonization in broilers. Campylobacteriosis is the most commonly reported bacterial foodborne zoonosis worldwide, and poultry products are the number one origin of these bacteria for human infection. To date, no effective control measures exist to limit *Campylobacter* colonization in the chicken's intestinal tract. Here, the effect of lyophilization of hyperimmune egg yolk on protection of broilers against *C. jejuni* was investigated. During an in vivo trial, broiler chickens were prophylactically given feed with lyophilized hyperimmune or non-immunized egg yolk powder starting from day 1 after hatch. At day 11, broilers were inoculated with *C. jejuni* according to a seeder model. Five days later, all broilers were euthanized and cecal content was examined for *C. jejuni* colonization. No decrease in *C. jejuni* colonization was found. The freeze-drying resulted in a 16-fold decrease of the antibody titer in the yolk powder compared to the fresh yolks, presumably caused by structural changes in the antibodies. In conclusion, applying freeze-dried hyperimmune egg yolk failed to protect broilers against *C. jejuni* colonization, possibly because lyophilization affected the antibodies' functionality.

Key words: *Campylobacter*, broiler, passive immunization, lyophilization, bacterin

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INTRODUCTION

Oral administration of antibodies is a promising strategy for controlling enteric bacterial and viral infections in humans and animals, such as *Salmonella enterica*, *Escherichia coli*, *Campylobacter jejuni*, *Clostridium perfringens*, rotaviruses, and coronavirus (Mine and Kovacs-Nolan, 2002; Schade et al., 2005; Chalghoumi et al., 2009a; Yegani and Korver, 2010; Gadde et al., 2015; Hedegaard and Heegaard, 2016). A cost-efficient method to produce such antibodies is through hyperimmunization of chickens and collecting the egg yolks, containing high amounts of antibodies (Bizanov, 2017).

We recently demonstrated that passive immunization using bacterin-induced antibodies was able to reduce *C. jejuni* infection in broilers (Vandeputte et al., 2019a). *Campylobacter* infection is the most commonly reported bacterial foodborne zoonosis in the European Union since 2005 (EFSA, 2017) and is mainly derived from poultry products. In most cases, clinical symptoms such as fever and diarrhea are self-limiting. However, complications may occur, such as reactive arthritis (Hannu et al., 2002) and Guillain-Barré syndrome (Nachamkin, 2002). Unfortunately, no effective measures to control *Campylobacter* infection in poultry exist to date (Hermans et al., 2011b). In our previous study (Vandeputte et al., 2019a), vaccination of layer hens resulted in a high and prolonged immune response, observed as the presence of high amounts of anti-*Campylobacter* IgY in the egg yolk. This hyperimmune yolk was administered to the feed of the broilers at a

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[1]Corresponding authors: Jasmien.Vandeputte@Ugent.Be (JV); An.Garmyn@Ugent.Be (AG)

concentration of 5%, resulting in a decrease of infected birds from 78 to 15%. However, under field conditions, egg yolk as such cannot be implemented and therefore an alternative administration method for these antibodies should be developed.

Lyophilization of egg yolk results in an easy-to-mix egg yolk powder (**EYP**) with an extended shelf-life. Moreover, the yolk is considered to form a protective matrix for the antibodies during their passage through the gastrointestinal tract (Schade et al., 2005) and to contain antimicrobial components (Kassaify and Mine, 2004a,b), so it would be advantageous to preserve these beneficial characteristics. Here, we investigated if we could obtain a colonization reduction similar to our previous results using hyperimmune EYP.

MATERIALS AND METHODS

Experimental Animals

Commercial Ross 308 broiler chickens of both sexes were purchased at a local hatchery (Vervaeke-Belavi, Tielt, Belgium). The animals were provided with a commercial feed and water ad libitum. Husbandry, experimental procedures, euthanasia methods, and biosafety precautions were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium, and in accordance with the relevant guidelines and regulations (EC2016/28). Birds were proved to be free of *Campylobacter* by examination of mixed fecal samples using standard methods as described by Hermans et al. (2011a).

Bacterial Strains and Culture Conditions

For the experimental infection, *C. jejuni* reference strain KC40 from poultry origin was used, which colonizes chickens to a high level (Van Deun et al., 2008). Bacteria were routinely cultured in Nutrient Broth No.2 (NB2, CM0067; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with Modified Preston *Campylobacter*-selective supplement (SR0204 E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232 E; Oxoid), at 42°C for 17 h under microaerobic conditions (5% O_2, 5% CO_2, 5% H_2, 85% N_2). *C. jejuni* bacteria in the broth were enumerated by plating tenfold dilutions in Hank's Balanced Salt Solution (**HBSS**; GIBCO-BRL, Invitrogen, Carlsbad, CA) on modified charcoal cefoperazone deoxycholate agar (**mCCDA**; CM0739; Oxoid) supplemented with CCDA selective supplement (SR0155 E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232 E; Oxoid), followed by microaerobic incubation at 42°C for 22 h.

Preparation of Hyperimmune Egg Yolk Powder

Hyperimmune egg yolks against a bacterin mix of 13 genetically diverse *C. jejuni* and *C. coli* strains and egg yolks from sham-immunized hens were previously produced by Vandeputte et al. (2019a) and further processed at the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO, Melle, Belgium). Before lyophilization, the yolks were stored at 4°C. The yolks were frozen at −50°C during 2 h and then lyophilized (sublimation: 0.16 mbar, 20 h −50°C to 20°C, 20 h at 20°C; desorption: 0.025 mbar, 2 h 20°C to 30°C, 3 h at 30°C; condenser temperature: −90°C) in an Epsilon 2-10 D LSC freeze-drier (Martin-Christ, Osterode am Harz, Germany). The hyperimmune and non-immunized EYP (resp. **HEYP** and **NEYP**) was stored at 4°C until further processing.

Prophylactic Efficacy of In-Feed Supplementation of Bacterin-Derived HEYP on C. jejuni Cecal Colonization in Broilers

Fifty-four day-of-hatch *Campylobacter* free broilers were raised in 2 randomly assigned treatment groups (n = 27/group) and housed in separate isolation units. From the day of hatch until the end of the experiment, the chicks were provided with feed containing 2.5% (wt/wt) HEYP (group 1) or NEYP (group 2), mixed manually through the feed. This concentration approaches the 5% egg yolk content used by Vandeputte et al. (2019a). Groups-administered fresh hyperimmune and non-immunized yolk were not repeated for ethical reasons, to reduce the number of animals. Equal amounts of feed and drinking water were provided for each group during treatment and care was taken that all animals had unlimited access to the feed and water. At 10 D of age, the chicks of each group were randomly assigned to 3 subgroups (n = 9/subgroup) and housed in separate isolation units. At 11 D of age, 3 seeder chicks of each subgroup were randomly selected and orally inoculated with approximately 1×10^5 cfu of *C. jejuni* strain KC40, quantified by plating as described previously. The birds that were not inoculated are referred to as sentinels. At day 16, all animals were euthanized by injection of an overdose (100 mg/kg) sodium pentobarbital (Kela, Hoogstraten, Belgium) in the wing vein, and the cecal content was collected for *C. jejuni* enumeration (as described in the following).

Cecal C. jejuni Enumeration

Cecal *C. jejuni* enumeration was performed by qPCR as described by Vandeputte et al. (2019b), originally adapted from Lund et al. (2004) and Botteldoorn et al. (2008). Briefly, DNA-extraction was performed using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions, with the single adaptation that the DNA was eluted in 100 µL instead of 200 µL ATE buffer. The DNA was stored at −20°C until further analysis.

Per qPCR reaction, 12.5 µL IQ Supermix (Bio-Rad, Temse, Belgium), 0.25 µL of each primer (forward primer Camp2F: 5′ CACGTGCTACAATGGCATAT

3′, reverse primer Camp2R: 5′ GGCTTCATGCTCTCGAGTT 3′), 0.25 μL probe (Camp2P: 5′ 6FAM-CAGAGAACAATCCGAACTGGGACA-BHQ1 3′), 6.75 μL HPLC-water, and 5 μL sample DNA were mixed until a total volume of 25 μL. Primers and probe were purchased at Integrated DNA Technologies (IDT; Leuven, Belgium). After centrifuging for 1 min at 1,500 tpm, the following qPCR program was run: 2 min at 50°C, 10 min at 95°C and 50 cycli of 15 s at 95°C and 60 s at 60°C (CFX96 Real-Time PCR Detection System, Bio-Rad). The number of *C. jejuni* in the cecal content was expressed as genomic equivalents (**ge**)/g cecal content.

Determination of Egg Yolk IgY Titers

Campylobacter-specific IgY titers in egg yolks were determined before and after lyophilization, as previously described by Hermans et al., (2014) with minor changes to the protocol. Egg yolks were diluted 1/5 (vol/vol) in HBSS, mixed thoroughly, and incubated overnight at 4°C. Lyophilized egg yolks were first diluted 1/2 (wt/vol) in HBSS because half of the egg yolk consists of water (Bizanov, 2017). The supernatant, containing the water-soluble fraction of the egg yolk, was collected for IgY quantification using ELISA. To determine egg yolk IgY titers, 96-well flat bottom plates (Nunc MaxiSorp, Nalge Nunc Int., Rochester, NY, USA) were coated (24 h, 4°C) with 10^6 cfu (before killing) bacterin diluted in a 50 μL coating buffer (2.16 g $Na_2CO_3.10H_2O$, 1.935 g $NaHCO_3$ in 500 mL H_2O). After washing (3× HBSS, 1× washing buffer: 0.1% Tween-20 in PBS), the wells were blocked (1 h, room temperature) with a 100 μL blocking buffer (1% BSA in washing buffer). Next, 100 μL of a 1/2 dilution series of the supernatant of the mixed egg yolks was incubated during 60 min at room temperature. Plates were washed as described previously and incubated with 100 μL 1/10,000 horseradish peroxidase–labeled anti-chicken IgY (Sigma Aldrich) in a washing buffer during 90 min at room temperature. After washing as described previously, the plates were incubated with 50 μL 3,3′,5,5′-tetramethylbenzidine substrate (Sigma Aldrich) for 10 min at room temperature in the dark. Next, 50 μL 0.5 M H_2SO_4 was added to each well and the absorbance at 450 nm ($\mathbf{OD_{450}}$) was measured using an automated spectrophotometer (Pharmacia LKB Ultrospec III, Gemini BV, Apeldoorn, Netherlands). IgY titers from yolks of immunized hens were reported as the highest dilution where the OD_{450} was greater than the OD_{450} + 3 standard deviations of wells containing yolk originating from sham-vaccinated birds.

Statistical Analysis

Data of the in vivo trial were analyzed using R 3.3.1. Before statistical analysis, *C. jejuni* counts were transformed to $\log_{10}$ counts. The colonization data were analyzed using a hurdle model, a class of models that assumes that the data are generated by 2 processes. First, the event that an individual is colonized (i.e., returning a non-zero count) follows a Bernoulli distribution. Given colonization, its intensity or load is a random variable following a discrete or continuous distribution; in this case, a gamma distribution was assumed. The model was used to estimate the probability of *C. jejuni* colonization and the mean *C. jejuni* numbers in the cecal content of colonized birds for each treatment level. Next, the pairwise differences between those and the proportion of the respective posterior distributions that had the same sign as the mean were calculated. If working in a null-hypothesis significance testing framework, this can be interpreted as a one-sided test, estimating the probability that the true difference between treatments is zero or greater (if negative) or smaller (if positive), and thus the level of confidence that the null hypothesis can be rejected. For a detailed description of the implementation in R, reference is made to Vandeputte et al., (2019a).

RESULTS

Protective Effect of Prophylactic Passive Immunization of Broilers With Bacterin-Derived HEYP Against Cecal C. jejuni Colonization

C. jejuni counts per gram cecal content after euthanasia of the chickens, prophylactically fed HEYP or NEYP, are given in Figure 1. No significant reduction of the number of *C. jejuni* colonized broilers was observed in HEYP-treated subgroups compared to the control subgroups (resp. 21/27, 24/27; $P > 0.05$). In each subgroup of both treatments, all seeders were colonized. The difference in the number of colonized birds between the 2 groups was a consequence of the variation in *C. jejuni* transmission to the sentinel birds. Yet, this reduction was not significant (resp. 12/18, 15/18;

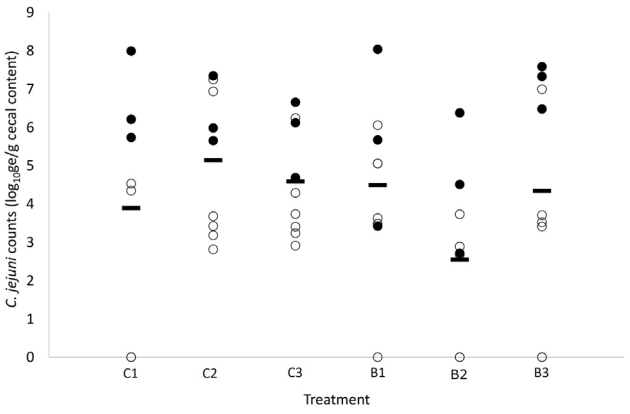

Figure 1. Individual and mean (−) cecal *C. jejuni* counts of colonized broiler seeders (•) and sentinels (○) after prophylactic treatment with lyophilized hyperimmune egg yolk. The birds received standard feed supplemented with 2.5% (wt/wt) lyophilized egg yolk from either sham-immunized (C1, C2, C3) or bacterin-immunized (B1, B2, B3) layers, from day 1 until day 16 (the day of euthanasia). At 11 D of age, seeder birds were inoculated with approximately 10^5 cfu *C. jejuni* KC40. Values are represented as $\log_{10}$ ge/g cecal content.

$P > 0.05$). No significant decrease of the mean *C. jejuni* counts in birds positive for colonization between the HEYP-treated and control groups was found [resp. 4.87, 5.11 $\log_{10}$ (ge/g cecal content); $P > 0.05$], as well as for seeders separately [resp. 5.79, 6.26 $\log_{10}$ (ge/g cecal content); $P > 0.05$] or sentinels separately [resp. 4.18, 4.41 $\log_{10}$ (ge/g cecal content); $P > 0.05$].

Effect of Hyperimmune Egg Yolk Lyophilization on Antibody Titers

The bacterin-induced *Campylobacter*-specific IgY titer in the egg yolks before lyophilization was 1:65,536, as determined by ELISA. After lyophilization, the antibody titer was reduced to 1:4,096.

DISCUSSION

In the present study, no protection against *C. jejuni* colonization was detected in chickens fed HEYP compared to control animals. This is in contrast to the results of Hermans et al., (2014) and Vandeputte et al., (2019a), the latter applying the same vaccines and procedures as used in this study, but using fresh yolks. A possible explanation is that lyophilization resulted in a 16-fold decline of the *Campylobacter*-specific antibody titer in the yolk, as determined by ELISA. Previously, Shimizu et al., (1988) found no effect of lyophilization on IgY activity, whereas others showed that freeze-drying resulted in some loss of antigen-binding activity of IgY (Sunwoo et al., 2002). Lyophilization of proteins induces freezing and dehydration stresses, which may result in protein structural changes or even unfolding (Emami et al., 2018). Therefore, to protect the proteins against these stresses, these authors recommend adding cryoprotectants and lyoprotectants.

As noted by Chalghoumi et al., (2009b) and Paul et al., (2014), the initial antibody dose, when orally administered, should be sufficiently high to ensure that an adequate amount of functional antibodies survives the gastrointestinal passage to establish protection against bacterial colonization. It is possible that the remaining titer after lyophilization (> 1:4.096) was insufficient compared to the titers supplemented by Vandeputte et al., (2019a) using fresh yolks (> 1:64.536).

The need to protect the antibodies during the passage through the gastrointestinal tract, against, for example, the acidic stomach environment and proteases, has been emphasized before (Schade et al., 2005; Chalghoumi et al., 2009a). Egg yolk is considered to form a protective matrix for the antibodies against degradation and functionality loss during this passage (Schade et al., 2005), but this protection might be affected during the freeze-drying process. To further investigate this hypothesis, further research will be needed, for example, determination of the effect of fresh yolk and lyophilized antibodies on the adherence of *C. jejuni* to intestinal mucus, because it was demonstrated previously that *Campylobacter*-specific antibodies enhance mucosal binding (Hermans et al., 2014). Other methods can be applied to increase stability of the antibodies, such as microencapsulation; however, this would increase the production complexity and cost (Chalghoumi et al., 2009a).

In conclusion, it was demonstrated that administering freeze-dried hyperimmune yolk did not protect broilers against *C. jejuni* colonization, in contrast to a previous study using fresh yolks. This is correlated with decreased antigen binding after lyophilization and may be further compromised by degradation during gastrointestinal passage. Further research will be needed to develop an industrially applicable method to administer these antibodies.

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