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Evaluation of the immune-stimulatory effects of plant essential oils using an infectious laryngotracheitis virus vaccination - challenge model

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Project Summary

Project Title	Evaluation of the immune-stimulatory effects of plant essential oils using an infectious laryngotracheitis virus vaccination - challenge model
Project No.	18-427
Date	Start: End: 30 March 2020
Project Leader(s)	Mauricio Coppo, Andres Diaz-Mendez, Joanne Devlin, Twan van Gerwe
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Project Aim	To determine the immune-modulatory effects of nutritional supplements using an infectious laryngotracheitis virus vaccination-challenge model in chickens.
Background	Infectious laryngotracheitis (ILT) is a significant problem for poultry producers in Australia. The disease is controlled by the application of biosecurity measures and live attenuated vaccines, which can prevent the appearance of clinical signs of disease but not re-infection with field strains or viral shedding. Plant oil extracts, such as eucalyptus or peppermint essential oils, and yeast products are known to stimulate innate immune responses and activate key mediators of the anti-viral response. Immune-modulation at vaccination by the administration of plant essential oils or soluble yeast may provide a way to prevent the disadvantages associated with vaccination while enhancing protection against subsequent re-infections.
Research Outcome	The administration of plant essential oils increased the safety characteristics of the vaccine tested but did not alter the efficacy of the immune responses involved in protecting chickens against challenge with virulent ILTV. In addition, the administration of plant essential oils during the first weeks of life resulted in significantly higher body weight gain over the duration of this study. The administration of soluble yeast was not associated with alterations of vaccine safety or efficacy, or body weight gain.
Impacts and Outcomes	Data from this study indicated that the administration of plant essential oils have positive effects on vaccine safety as indicated by an increase in body weight gain after vaccination and reduced viral infection in sites of the respiratory tract. These results suggest that plant essential oils have immune-modulatory effects *in vivo*, which result in enhanced anti-viral responses. These enhanced anti-viral effects may be beneficial in the containment of other viral pathogens that infect chickens during early stages of their life.
Publications	In preparation.

Executive Summary

Infectious laryngotracheitis (ILT) is a significant problem for poultry producers in Australia. The disease is controlled by the application of biosecurity measures and live attenuated vaccines. These vaccines can prevent the appearance of clinical signs of disease but not re-infection with field strains or viral shedding. In addition to this, live attenuated vaccines can have high levels of residual virulence, which, in some cases, can increase further after bird-to-bird passage. Therefore, vaccine strains that are shed in high levels or are prone to transmission between birds are relatively more likely to cause 'vaccinal ILT' outbreaks or lead to recombination events. Plant essential oil extracts, such as eucalyptus or peppermint essential oils, and yeast products are known to stimulate innate immune responses and activate key mediators of the anti-viral response. Immune-modulation at vaccination by the administration of plant essential oils or soluble yeast may provide a way to prevent the disadvantages associated with vaccination while enhancing protection against subsequent re-infections. The aim of this project was to determine the immune-modulatory effects of nutritional supplements using an infectious laryngotracheitis virus vaccination-challenge model in chickens. Six groups of specific-pathogen-free (SPF) chickens received no nutritional supplements (groups 1-3), or received plant essential oils from mint and eucalyptus in the drinking water between hatch and 17 or 35 days of age (groups 4 and 5, respectively), or soluble yeast (group 6) from hatch until 35 days of age. At 7-days of age, chickens in groups 3-6 were vaccinated with Poulvac Laryngo A20 via eye-drop. Four days after vaccination (11-day old), 5 chickens in each of groups 2-6 were euthanased and sampled. At three weeks after vaccination (28-days of age), chickens in groups 2-6 were challenged with virulent ILTV via the intra-tracheal route. Clinical signs were scored four days after challenge (32-day old). One week after challenge (35-day old), all chickens were euthanased and sampled. Weight data was collected at 6, 11 and 35 days of age.

Samples collected included conjunctival, palatine cleft and tracheal swabs for molecular detection of viral DNA and host RNA, and upper tracheal sections for histopathology. The administration of plant essential oils increased the safety characteristics of the vaccine tested. Chickens treated with the essential oils did not decrease in weight after vaccination and had lower levels of viral shedding from sites of the upper respiratory tract. These effects after vaccination did not alter the efficacy of the immune responses involved in protecting chickens against challenge with virulent ILTV. In all groups the vaccine was highly protective. In addition, the administration of plant essential oils during the first weeks of life resulted in significantly higher body weight gain over the duration of this study. The administration of soluble yeast was not associated with alterations of vaccine safety or efficacy, or body weight gain. Data from this study demonstrated that the administration of plant essential oils has positive effects on vaccine safety, indicated by increased body weight gain after vaccination and reduced viral infection in sites of the respiratory tract. These results suggest that plant essential oils have immune-modulatory effects *in vivo*, which result in enhanced anti-viral responses. These enhanced anti-viral effects may be beneficial in the containment of other viral pathogens that infect chickens during early stages of their life.

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Introduction

The increasing awareness of the threat to human and veterinary public health that antimicrobial resistance represents has put pressure on animal production systems to revisit antimicrobial use guidelines, thus reducing or even abolishing the use of antimicrobials. Consequently, the optimisation

of strategies to prevent infectious diseases in animal production systems has gained renewed attention. Such strategies have traditionally been focussed on biosecurity, vaccination, genetic selection for traits associated with resistance to disease, and more recently on nutritional interventions to improve gut health and function through the modulation of enteric microbial populations. In addition to this, there is a focus on the use of nutritional products with immune-modulating capacities that can enhance the response of individuals to vaccination or infection.

Accumulating evidence in the literature has indicted that plant oil extracts, such as those obtained from eucalyptus (*Eucalyptus globosus*) and mint (*Mentha spp.*) can have immune-modulatory functions *in vitro* and *in vivo*. Eucalyptus and mint essential oils are composed of a variety of monoterpenoid compounds, with 1,8-cineole (eucalyptol) and L-menthol being the most abundant in each, respectively. Other components include α-pinene, β-pinene, α-phellandrene, δ-limonene in eucalyptus oil; and L-menthone, menthyl acetate, 1,8-cineole, limonene, β-pinene and β-caryophyllene in mint oil. Both types of essential oils have the capacity to stimulate macrophages or macrophage cell lines to increase their phagocytic activity, while simultaneously suppressing inflammation (1-7). While eucalyptol has shown a strong Th2 inclination and anti-inflammation potential *in vitro* when applied on mouse primary splenocytes, menthone may have a relative Th1-inclination property (4). In addition, eucalyptus oil has mucolytic and spasmolytic effects in the respiratory tract, and its use in human medicine has been beneficial in the management of chronic obstructive pulmonary disease and asthma. This is possibly due to its anti-inflammatory and anti-oxidant mode of action (3).

In chickens, the available literature is quite limited. The combined use of eucalyptus and peppermint essential oils has been tested as an immune-modulator during vaccination against Newcastle Disease virus with positive effects, where treatment with essential oils resulted in increased body weight gain and survival after challenge, as well as high antibody and haemmagglutination titres (2). There is also evidence that essential oils of eucalyptus and peppermint, used in combination with vaccination against avian influenza, significantly increased the antigen-specific antibody titres following immunisation. Although the exact mechanism behind this effect in chickens is unknown, it may be associated with an increased phagocytic activity of macrophages (1). Also, when applied in the diet of female broilers, peppermint essential oil had positive effects in terms of growth performance under heat stress conditions (8).

There is also evidence for a significant immune-modulatory role of yeast and yeast-derived products in chickens (9-12). Previous studies have investigated the effects of adding yeast cell wall, nucleotides or a combination of both in the diets of broiler chickens (9-12), and determined that the addition of yeast products to the diet had significant impacts on the transcription of pathogen recognition receptors (PRR) such as toll-like receptors (TLR) and macrophage mannose receptor (MMR), and cytokines, both at a

local level in the intestinal tract and/or at a systemic level in the spleen. Subsequent challenge or stimulation with *Escherichia coli* lipopolysaccharide (LPS) (11), *Clostridium perfringens* (12), sheep red blood cells, or bovine serum albumin (9, 10) resulted in enhanced immune responses. As an example, broiler chickens fed yeast-cell wall displayed higher gene transcription levels of cytokines and PRR in spleens, following challenge with *E. coli* LPS. This was especially noticeable for TLR21 and MMR, as well as for interleukin (IL)-6 and IL-8, interferon (IFN)-γ and IL-12, and IL-10 and IL-4. There was also an increase in local IgA titres in these chickens (11). One of these studies also identified that diets containing yeast cell wall extracts had a Th2 biased immune response, driven by upregulation of IL-4, IL-13 and IL-10 (9).

Considering the above, this project proposed the hypothesis that nutritional supplements containing plant essential oils, or yeast-derived products, would have positive impacts on the immune responses induced following inoculation with an attenuated viral vaccine or after challenge with virulent virus.

There are number of features of the infectious laryngotracheitis virus (ILTV) model that make it suitable for use in this context, including: i) ILTV causes significant morbidity and mortality in commercial poultry and advancements in methods to enhance the outcomes of vaccination strategies would be desirable; ii) a well-established vaccination-challenge model developed in our laboratory is readily available and; iii) the potential for immediate application of research outcomes under field conditions.

Objectives

The aim of this project was to determine whether the simultaneous application of ILTV vaccine and nutritional supplements containing plant essential oils, or yeast-derived products, affects the outcomes of infection following vaccination with a commercial vaccine and challenge with virulent virus. More specifically, this project aimed to determine:

1. The safety characteristics of an ILTV vaccine, whilst chickens were treated with plant essential oils or yeast-derived products in the drinking water.
2. The outcomes of infection after challenge with virulent ILTV, in chickens that were treated with plant essential oils or yeast-derived products in the drinking water at the time of vaccination but not during challenge.
3. Determine the outcomes of infection after challenge with virulent ILTV, in chickens that were treated with plant essential oils or yeast-derived products in the drinking water at the time of vaccination and during challenge.

All these objectives were met.

Methodology

Viruses

The commercial ILTV vaccine Poulvac® Laryngo A20 (Zoetis) was used in the current study. Each commercial label dose contains at least $10^{3.5}$ pock forming units of the Australian A20 ILTV vaccine strain. The A20 ILTV vaccine strain was generated by sequential passages of the Australian SA2 ILTV strain in chicken embryos and cultured cells (13).

The Australian recombinant virulent class 10 ILTV strain was used as a challenge strain in the current study. This virulent ILTV field strain emerged in Australia by spontaneous recombination between Australian and European vaccine strains (14) and is highly virulent in SPF chickens (15).

Nutritional supplements

Two commercial nutritional supplements, Grippozon® (EW Nutrition GmbH) and LiquiPro™ SC (Diamond V Mills Inc.), were tested in the current study for their immune-modulatory properties. Grippozon® contains eucalyptus (*Eucalyptus globulus*) and mint (*Mentha arvensis*) oil. LiquiPro™ SC is the liquid product derived from the fermentation of *Saccharomyces cerevisiae*, including the products of fermentation, residual yeast cells and yeast cell fragments as well as remaining media following fermentation. Both products were administered in the drinking water of experimental birds.

***In vivo* experimental design**

This *in vivo* study was approved by the Faculty of Veterinary and Agricultural Sciences Animal Ethics Committee (AEC ID#1814710.1-3), The University of Melbourne, in accordance with the Australian Code for the Responsible Conduct of Research and the Australian Code for the Care and Use of Animals for Scientific Purposes.

A summary of the experimental groups is shown in Table 1. One-hundred and fifteen 1-day-old Leghorn-type specific-pathogen-free (SPF) chickens were wing-tagged for identification purposes and randomly allocated to five groups of 20 birds, or one group of 15 birds. Each group of birds was housed in a separate Horsfall-Bauer type isolator and fed irradiated feed *ad libitum*. Birds in groups 4 and 5 were provided with water supplemented with the commercial product Grippozon at the concentration recommended by the manufacturer of 0.02% ($^{v}/_{v}$). Birds in group 6 received water supplemented with LiquiPro™ SC at the concentration recommended by the manufacturer of 0.1% ($^{v}/_{v}$). Birds in group 4 received the supplemented water between 1 and 17 days old. Birds in groups 5 and 6 received the supplemented water between 1 and 35 days old.

Table 1. Experimental groups.

Group	n	Nutritional supplement				Vaccination				Challenge			
		Product	Dose (% $^{v}/_{v}$)	Route	Period (days)	Vaccine	Dose (PFU)	Route	Age (days)	ILTV strain	Dose (PFU)	Route	Age (days)
1	15	Nil	NA	NA	NA	Nil	NA	NA	NA	Nil	NA	NA	NA
2	21	Nil	NA	NA	NA	Nil	NA	NA	NA	Class 10	$10^{3.0}$	IT	28
3	21	Nil	NA	NA	NA	A20	$10^{3.5}$	ED	7	Class 10	$10^{3.0}$	IT	28
4	22	Essential oils	0.02	Oral	1 – 17	A20	$10^{3.5}$	ED	7	Class 10	$10^{3.0}$	IT	28
5	21	Essential oils	0.1	Oral	1 – 35	A20	$10^{3.5}$	ED	7	Class 10	$10^{3.0}$	IT	28
6	22	Soluble yeast	0.1	Oral	1 – 35	A20	$10^{3.5}$	ED	7	Class 10	$10^{3.0}$	IT	28

A20: Poulvac® A20 (Zoetis)
PFU: Plaque forming units
ED: Eye-drop
IT: Intra-tracheal
NA: Not Applicable

All birds were weighed at 6-days-old, before vaccination. At 7 days-old, all birds in groups 3 – 6 were vaccinated with one commercial dose of the A20 vaccine via eye-drop inoculation. Each dose was administered in 25 µL of inoculation diluent (Dulbecco's modified Eagle's medium [DMEM] supplemented with 5 µg/mL of gentamicin, 5 µg/mL of ampicillin and 0.5 µg/mL of amphotericin B). At 4 days post-vaccination (pv), five birds in each of groups 2 - 6 were culled by exposure to halothane. During post-mortem examination, all birds were weighed before swabs were collected aseptically from the conjunctiva of both eyes, the palatine cleft and the trachea. Swabs were collected in 500 µL of viral transport media (DMEM supplemented with 4% foetal bovine serum, hydroxyethyl piperazineethanesulfonic acid (pH 7.7; HEPES), 500 µg/mL of gentamicin, 500 µg/mL of ampicillin and 50 µg/mL of amphotericin B) and stored on ice before permanent storage at -80 °C until processing for nucleic acid extraction.

An upper trachea section was collected from each bird. This tracheal section was fixed in 10% buffered formalin and processed for histopathological examination and scoring after haematoxylin and eosin staining. The sex of each chicken was recorded as well, to aid in the interpretation of weight gain and other data.

At 28 days of age (21 days pv), all birds in groups 2 - 6 were challenged with 10^3 plaque forming units (PFU) of the virulent Australian class 10 ILTV strain (14, 15). Challenge virus was administered in 150 µL of inoculation diluent via the intra-tracheal route, using an 18G gavage needle as previously described (15). Clinical signs of ILT were scored at 4 days post-challenge (pc) using a previously described scoring system (16), based on the assessment of demeanour (score 0 - 3), conjunctivitis (score 0 – 2) and dyspnoea (score 0 – 3). At 35 days of age (*i.e.*: 7 days pc), all chickens were culled by exposure to halothane. Culled birds were then weighed and subjected to post-mortem examinations and sampling. All swab and tissue samples were collected and stored as described above.

Nucleic acid extraction on swab samples

Nucleic acids were extracted using 200 µL of each swab sample, the X-tractor system (Corbett Robotics) and the VX Universal Liquid Sample DNA extraction kit (Qiagen) as per manufacturer's instructions. Twelve negative extraction controls and at least one positive extraction control were included in each run. Elutions were made in 50 µL of Tris EDTA. 20 µL of each eluate were subjected to DNase treatment using the Turbo DNA-free kit (Ambion) as per manufacturer's instructions and stored at -20 °C until reverse transcription and qPCR.

qPCR analysis for determination of viral DNA in swabs

Extracted nucleic acid samples were subjected to qPCR using a previously described set of oligonucleotide primers (Table 2) that amplify a 113 bp fragment of the UL15 gene (17). Triplicates of

ten-fold dilutions of the amplicon in pGEM®-T (Promega) ranging from 10^8 to 10^1 gene copy numbers (GCN) per reaction were used to generate a standard curve in each run. A no template control was included in each run. Samples with GCN equal or higher than 100 per reaction (Ct value > 29) and high-resolution melt curve profiles consistent with those of the positive control samples were regarded as positive. Genome copy number (GCN) values were Log_{10} transformed before statistical analyses. The cut-off value for the assay was 200 copy numbers. All negative samples were assigned a random value between 0 and of 200 GCN before Log_{10} transformation for statistical analyses.

Reverse transcription and qPCR analysis for determination of host gene transcripts

Nucleic acid samples subjected to DNase treatment were reverse-transcribed to generate complementary DNA (cDNA) using Superscript™ III (Invitrogen™), RNaseOUT ™ (Invitrogen™) and 2.5 μM Random Hexamers (Invitrogen™) as per manufacturer's instructions. A separate set of aliquots of nucleic acid samples subjected to DNase treatment was used as a no reverse transcription control. Samples containing cDNA were used to determine the transcription of a panel of six host immune genes (interferon gamma [IFN-γ], interleukin[IL]-12p40, IL-18, tumour necrosis factor alpha [TNF-α], granzyme-A [GZM-A], and chicken CXC chemokine ligand inflammatory 2 [chCXCLi2]), which were normalised against 18S ribosomal RNA (18S-rRNA). Table 2 contains details about all the oligonucleotide primers used in this study. Triplicate samples of serial 10-fold dilutions containing 10^8 – 10^1 copy numbers per reaction of each amplicon in pGEM®-T (Promega) were used to generate standard curves for each qPCR in order to estimate amplification efficiency and determine Cycle threshold (Ct) values for test samples. Al qPCR reactions were performed in a Rotor-Gene Q thermocycler (Qiagen) and fluorescence data was acquired and analysed using the Rotor-Gene Q Software (v2.1.0; Qiagen). Mean Normalised Expression (MNE) was used to calculate the transcription of host immune genes relative to 18S-rRNA (18, 19).

Histopathological assessment and scoring

Tracheal sections fixed in 10% buffered formalin were subjected to standard histological processing, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Stained sections were assessed under a light microscope and histopathological lesions were scored using a previously described method (20). This scoring system ranges from 0 to 5 and is based on the assessment of a series of parameters including mucosal thickness and oedema, presence of perivascular cuffs, leukocyte infiltration, hyperaemia, damage of the epithelium, and observation of syncytia and intra-nuclear inclusion bodies.

Table 2. Oligonucleotoide primers used for viral detection and host gene transcription analyses using quantitative polymerase chain reaction.

Gene target	Primer orientation	5' – 3' sequence	Amplicon size (bp)	Annealing temperature (°C)	$MgCl_2$ concentration (mM)	Reference
UL15	Forward	TTGCTGTGCTATTTCGCGTG	113	60	2	(17)
	Reverse	GTAAATCGTTTAGTGCGGCAT				
18S-ribosomal RNA	Forward	CATGTCTAAGTACACACGGGCGGTA	134	60	3	(21)
	Reverse	GGCGCTGCTGGCATGTATTA				
Interferon gamma	Forward	AGCTGACGGTGGACCTATTATT	259	55	1	(22)
	Reverse	GGCTTTGCGCTGGATTC				
Interleukin-12p40	Forward	AGACTCCAATGGGCAAATGA	274	58	3	(23)
	Reverse	CTCTTCGGCAAATGGACAGT				
Interleukin-18	Forward	GGAATGCGATGCCTTTTG	264	60	1	(22)
	Reverse	ATTTTCCCATGCTCTTTCTCA				
Tumour necrosis factor alpha	Forward	CGCTCAGAACGACGTCAA	115	60	3	(21)
	Reverse	GTCGTCCCACACCAACGAG				
Granzyme A	Forward	TGGGTGTTAACAGCTGCTCATTGC	453	60	3	(24)
	Reverse	CACCTGAATCCCCTCGACATGAGT				
CXC chemokine ligand inflammatory 2	Forward	ATGAACGGCAAGCTTGGAGCT	278	64	3	(25)
	Reverse	GCCATAAGTGCCTTTACGATCAG				

Statistical analyses

Clinical sign scores and tracheal histopathology scores were assessed using Mann-Whitney's U-test. This data was managed in Microsoft Excel and analysed using Minitab version 19.

Management of the qPCR data was conducted in Microsoft Excel and analysis in R (R Core Team, 2019). The qPCR results for the conjunctiva, palatine cleft, and trachea were each defined as the response variable in a general linear model. Models were fitted via maximum likelihood estimation. The data were divided by day of observation, under the expectation that the day 4 and day 7 results may have mechanistically different relationships to the predictors. The models were of the form:

Equation 1: $log_e(qPCR, d4) \sim 1 + Vaccine + Treatment + Sex$

Equation 2: $log_e(qPCR, d7) \sim 1 + Vaccine + Challenge + Treatment + Sex$

The presence of left-censoring of the responses at the limit of detection of the qPCR assay (200 copies), which was abundant in the dataset (246 of 345 observations) needed to be taken into consideration during our analyses (Uh et al., 2008). To obtain valid parameter and interval estimates, a multiple imputation (MI) approach was applied for these left-censored observations. At each MI iteration, each of the censored observations was replaced by a uniform value (Canales et al., 2018) on the interval (0, 200). The logarithmic transformation was then applied to the response variable and the models fitted as above.

The skewed distributions of the residuals in the fitted models also needed to be taken into consideration. As the predictors were exclusively categorical and a logarithmic transformation was already applied, no model modifications were readily available to accommodate non-normality of the residuals. As the primary research interest was in the treatment effect estimates and their uncertainty, a bootstrapping approach was applied to obtain robust interval estimates (DiCiccio and Efron, 1996; Pek et al., 2018). The non-parametric bootstrap was applied as implemented in the 'boot' package. To combine the MI and bootstrap simulations, the 'Boot MI' method (Schomaker and Heumann, 2018) was used, in which the multiple imputation (50 imputations) estimates were obtained within each of 2,000 bootstrap replicates, and the final 95% confidence intervals were determined from the bootstrapped distribution using the percentile method (Puth et al., 2015).

Changes in bodyweight after vaccination and at the end of the experiment were evaluated using a linear model, where the response was the total weight gained (final weight minus initial weight). The initial weight was included as a covariate (Kronmal, 1993). The model was of the form;

Equation 3: $$BW_{gained} \sim 1 + BW_{initial} + Vaccine + Challenge + Treatment + Sex$$

This model was assessed using the asymptotic 95% confidence intervals of the parameter estimates, the adjusted R^2, and the histogram of residuals.

Discussion of Results

Plant essential oils prevented decreased weight gain associated with vaccination.

Weight gain between 1 day before vaccination and 4 days post-vaccination were calculated and are summarised in Figure 1. The body weight gain model was a reasonable fit to the data, with an acceptable distribution of the residuals (Supplementary Figure 1) and R^2: 0.7313. Vaccination had a negative effect on body weight gain, whereas initial weight and supplementation with plant essential oils had positive effects (Supplementary Table 1).

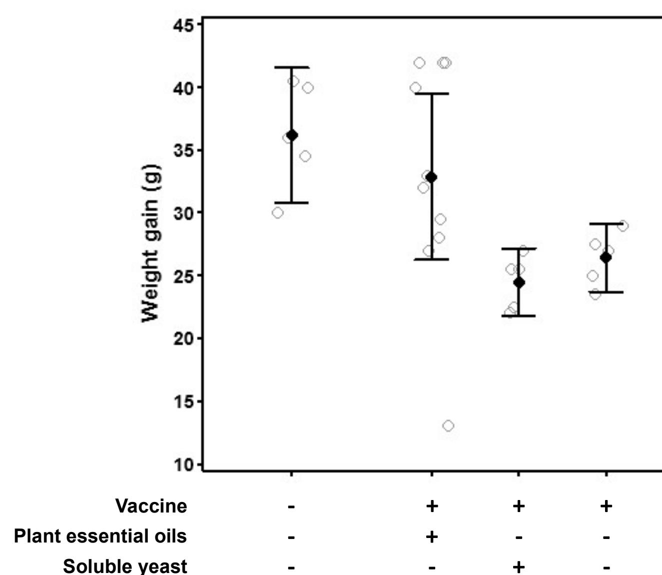

Figure 1. Individual, mean and 95% confidence interval weight gain of female (F) and male (M) specific-pathogen-free chickens between 1-day before (6-days-old) and 4-days after eye-drop vaccination with A20 vaccine. Chickens received plant essential oils, or soluble yeast in the drinking water, or remained unsupplemented from hatch until the weighing day.

Plant essential oils reduced vaccine ILTV detection in sites of the upper respiratory tract.

Conjunctival, palatine cleft and tracheal swab material collected at 4 days post vaccination was analysed for the presence of ILTV DNA using qPCR. These analyses revealed that eye-drop vaccination resulted

in infection of the conjunctiva and palatine cleft in most vaccinated birds, with no significant differences observed in the proportions of qPCR positives between the different groups of vaccinated birds (Table 4). This is consistent with conjunctiva being the inoculation site. However, significantly lower ILTV Log_{10} GCN were detected in palatine cleft swabs collected from birds that received plant essential oils compared to vaccinated birds that received no supplementation or soluble yeast in the drinking water (Figure 2, Supplementary Table 2). Much lower levels of ILTV GCN were detected in tracheal swabs across all groups of vaccinated birds. Among these tracheal samples, both the proportion of qPCR positives (Table 4) and the Log_{10} GCN detected in swabs collected from those that received plant essential oils were significantly lower compared to that of the other vaccinated groups of birds (Figure 2, Supplementary Table 2). Consistent with the very low level of ILTV GCN detected in tracheal samples from vaccinated birds, the histopathology scores in these groups of birds were not significantly different to those of unvaccinated chickens (Table 4).

Lower levels of ILTV detection in the palatine cleft and trachea in those that received plant essential oils, supports the hypothesis that enhanced anti-viral immune responses were induced in these chickens, resulting in reduced infection of the upper respiratory tract. It is unclear whether this enhanced anti-viral immune response prevented the spread of virus from the inoculation site to other sites of the upper respiratory tract, or if the virus did spread to these sites, but was then cleared more efficiently. To help answer this question, available swab samples from this time point were subjected to RNA extraction, DNase digestion and host gene transcription analysis. Except for the house keeping gene (18s-rRNA), which was detected in all samples ($1.39\times10^1 - 2.66\times10^6$ copy numbers per reaction), none were positive for the cytokine genes analysed, and very few samples were positive for the chCXCLi2 (IL-8) gene. This prevented statistical analysis and meaningful interpretation. It is possible that swab material contained insufficient cell numbers to quantify cytokine and chemokine gene transcription, despite the high levels of sensitivity of the RT-qPCRs used (10-100 copy numbers depending on the qPCR, data not shown). The same swab material was used successfully to detect ILTV DNA, emphasising the difficulties of working with RNA. Our previous studies have used tissue samples, rather than swab material, for host gene transcription analyses, and results from the current study favour the use of the latter.

Taken together, the weight gain and viral qPCR data from 4 days after vaccination indicates that chickens that drank water with plant essential oils were better protected against infection with a low virulence ILTV strain, compared to those that drank potable water with soluble yeast or water without further supplementation. Vaccination had a negative impact on weight gain, which was reversed by the intake of plant essential oils before, and during, the time of vaccination, indicating that the enhanced anti-viral response had a positive influence on the productive performance of treated chickens. It remains to be determined if the beneficial effects of the essential oils observed in the current study using

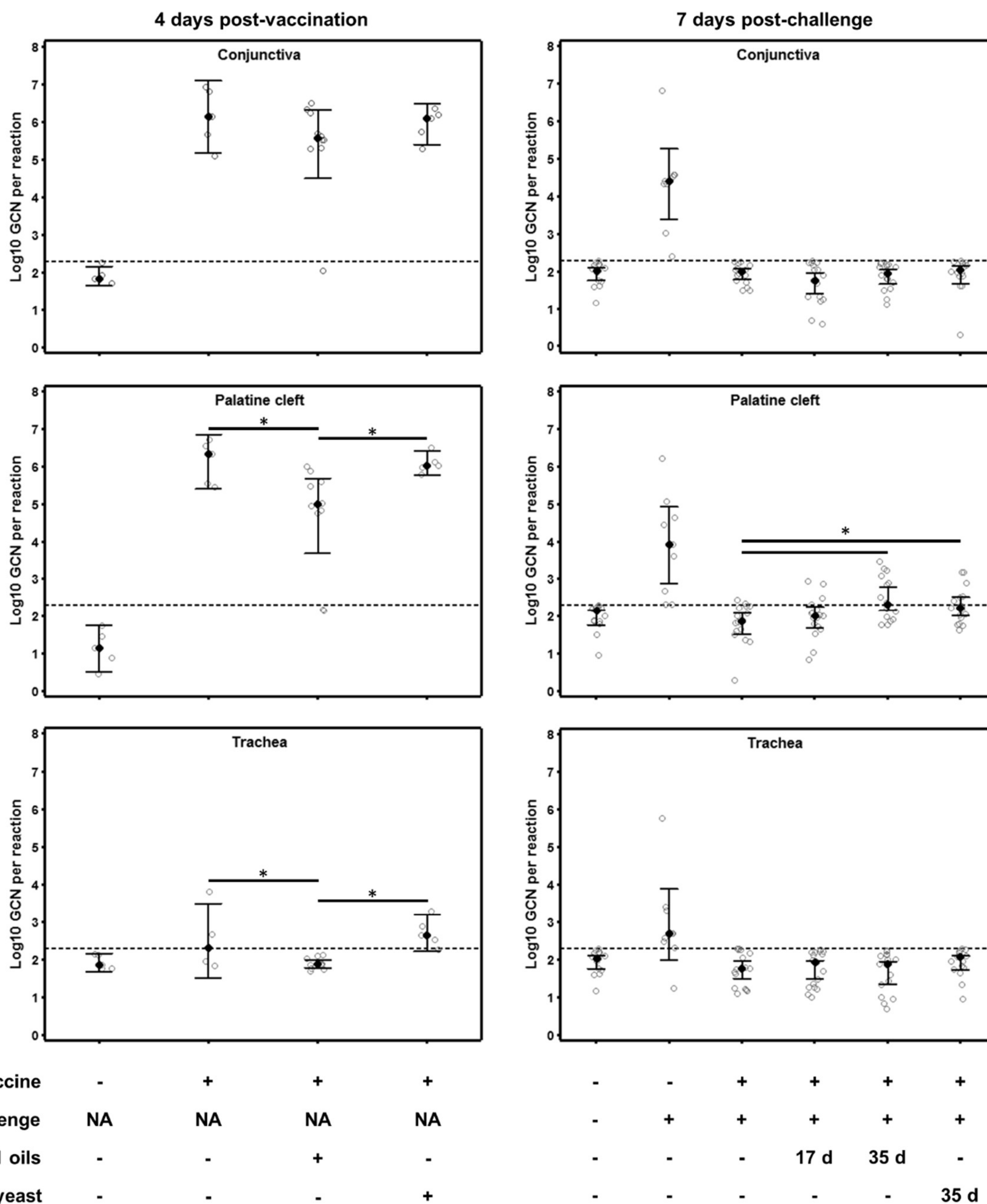

Figure 2. Individual, mean and 95% CI ILTV Log_{10} genome copy numbers (GCN) per reaction detected in conjunctival, palatine cleft or tracheal swabs collected from specific-pathogen-free chickens 4 days after eye-drop inoculation with A20 ILTV vaccine, or 7 days after intra-tracheal challenge with virulent class 10 ILTV. Chickens had been receiving potable drinking water or water supplemented with plant essential oils for 17 and 35 days from hatch, or soluble yeast for 35 days from hatch. All samples with a qPCR negative result were assigned a random value between 0 and 2.30 Log10 GCN per reaction for statistical analyses. Bars and * indicate significant differences (as shown in Supplementary Tables 2 and 3) between groups of vaccinated- or vaccinated-and-challenged-birds at 4 or 7 days after vaccination or challenge, respectively.

Table 4. Proportion of qPCR positives detected in conjunctival, palatine cleft and tracheal swabs, and tracheal histopathology scores of upper tracheal sections collected from specific-pathogen-free chickens 4 days after eye-drop inoculation with ILTV vaccine.

Vaccine	Nutritional supplement†	qPCR positive proportion (95% CI)			Median tracheal histopathology score (range)
		Conjunctiva	Palatine cleft	Trachea	
Nil	Nil	0/5 (0.00-0.45) [a]	0/5 (0.00-0.45) [a]	0/5 (0.00-0.43) [a]	0 (0-1) [a]
A20	Nil	5/5 (0.55-1.00) [b]	5/5 (0.55-1.00) [b]	3/5 (0.15-0.95) [ab]	0 (0-0) [a]
A20	Essential oils	9/10 (0.55-0.99) [b]	8/10 (0.44-0.97) [b]	0/10 (0.00-0.28) [a]	0 (0-1) [a]
A20	Soluble yeast	5/5 (0.55-1.00) [b]	5/5 (0.55-1.00) [b]	4/5 (0.38-0.96) [b]	0 (0-0) [a]

[a, b] The same lowercase superscript character in each column indicates values were not statistically significantly different ($P > 0.05$).

Proportions of qPCR positives were compared between groups using Fisher's exact test.

Tracheal scores were compared between groups using Mann-Whitney's U test.

† birds drank potable water (Nil), or water containing essential oils or soluble yeast.

a highly attenuated vaccine strain, such as A20, may also be a feature during vaccination using less attenuated ILTV vaccine strains available in Australia.

Nutritional supplementation in the drinking water did not alter the level of protection against challenge. Three weeks after vaccination, all groups of vaccinated chickens and one group of unvaccinated chickens were challenged via the intra-tracheal route with virulent ILTV. All vaccinated birds were clinically healthy at 4 days after challenge, in contrast with unvaccinated birds which had mortality (Figure 3), showed severe clinical signs consistent with ILT (Table 5) and had severe histopathological lesions in tracheal sections (Table 6). Swab samples collected during post-mortem examination 7 days after challenge showed that intra-tracheal challenge with virulent ILTV resulted in significantly higher viral GCN per reaction in all examined tissues of the unvaccinated, compared to unchallenged control birds (Figure 2 and Supplementary Table 3). Vaccination was associated with a large decrease in the proportion of qPCR positives (Table 6) and viral Log_{10} GCN (Figure 2) detected in all sampling sites. Supplementation with plant essential oils or soluble yeast for 35 days had an apparent positive effect on GCN detection in the palatine cleft swabs, whereas male sex had a positive effect on viral GCN determined in conjunctival swabs at this same time point.

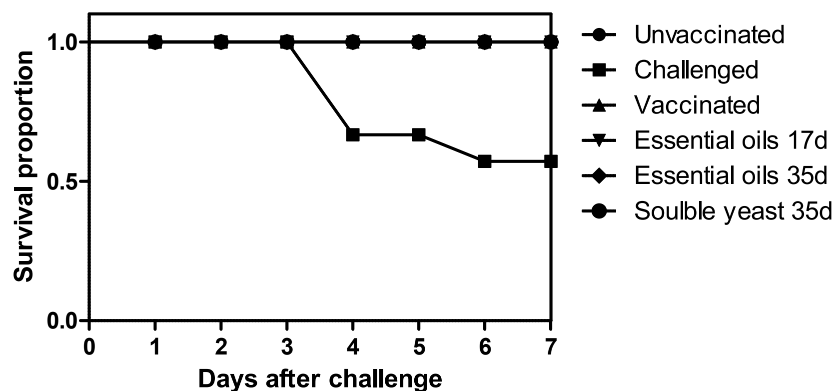

Figure 3. Survival of specific-pathogen-free chickens after challenge with virulent ILTV via the intra-tracheal route three weeks after vaccination. Chickens received either plant essential oils in the drinking water between hatch and 17 or 35 days of age, or soluble yeast between hatch and 35 days of age. Control groups included unvaccinated-unchallenged (Unvaccinated), unvaccinated-challenged (Challenged) and vaccinated-challenged (Vaccinated) without further supplementation.

The analysis of data and samples collected after challenge indicated that inoculation with the Australian recombinant Class 10 ILTV strain used in this study was associated with severe disease in unvaccinated chickens, causing mortality, severe clinical signs of ILT, severe tracheal histopathology, decreased weight gain and high levels of ILTV detection in sites of the respiratory tract and conjunctiva.

Table 5. Clinical sign scores assessed in specific pathogen free chickens 4-days after challenge with virulent ILTV via the intra-tracheal route.

Vaccine	Nutritional supplementation		Challenge strain	Median clinical sign scores (range)			
	Product given†	Age (days)		Demeanour	Dyspnoea	Conjunctivitis	Sum
Nil	Nil	NA	Nil	0 (0-0) [a]	0 (0-0) [a]	0 (0-0) [a]	0 (0-0) [a]
Nil	Nil	NA	Class 10	1 (0-2) [b]	2 (0-4) [b]	0 (0-0) [a]	3 (0-3) [b]
A20	Nil	NA	Class 10	0 (0-0) [a]	0 (0-0) [a]	0 (0-0) [a]	0 (0-0) [a]
A20	Essential oils	0 – 17	Class 10	0 (0-0) [a]	0 (0-0) [a]	0 (0-0) [a]	0 (0-0) [a]
A20	Essential oils	0 – 35	Class 10	0 (0-1) [a]	0 (0-0) [a]	0 (0-0) [a]	0 (0-0) [a]
A20	Soluble yeast	0 – 35	Class 10	0 (0-0) [a]	0 (0-0) [a]	0 (0-0) [a]	0 (0-0) [a]

[a, b] The same lowercase superscript character in each column indicates the clinical signs scores were not statistically significantly different ($P > 0.05$) between groups using Mann-Whitney's U test.

† birds drank potable water (Nil), or water containing plant essential oils or soluble yeast.

NA: Not Applicable

Table 6. Proportion of qPCR positives detected in conjunctival, palatine cleft and tracheal swabs, and tracheal histopathology scores of upper tracheal sections collected from specific-pathogen-free chickens 7 days after intra-tracheal inoculation with virulent ILTV.

	Nutritional supplementation		Challenge	qPCR positive proportion (95% CI)			Median tracheal
Vaccine	Product given†	Age (days)	strain	Conjunctiva	Palatine cleft	Trachea	histopathology score (range)
Nil	Nil	NA	Nil	0/15 [a] (0.00 – 0.18)	0/15 [a] (0.00 – 0.18)	0/15 [a] (0.00 – 0.18)	0 (0-1) [a]
Nil	Nil	NA	Class 10	9/9 [b] (0.72 – 1.00)	9/9 [b] (0.72 – 1.00)	8/9 [b] (0.52 – 0.99)	3 (3-4) [b]
A20	Nil	NA	Class 10	0/16 [a] (0.00 – 0.17)	3/16 [ac] (0.04 - 0.46)	0/16 [a] (0.00 – 0.17)	1 (1-2) [c]
A20	Essential oils	0 – 17	Class 10	0/17 [a] (0.00 – 0.16)	6/17 [cd] (0.14 - 0.62)	0/17 [a] (0.00 – 0.16)	2 (1-2) [d]
A20	Essential oils	0 – 35	Class 10	0/16 [a] (0.00 – 0.17)	9/16 [d] (0.29 – 0.80)	0/16 [a] (0.00 – 0.17)	1 (1-2) [ce]
A20	Soluble yeast	0 – 35	Class 10	0/17 [a] (0.00 – 0.16)	9/17 [d] (0.28 – 0.77)	0/17 [a] (0.00 – 0.16)	1 (1-2) [de]

[a, b, c, d, e] The same lower-case superscript character on each column indicates the proportions of qPCR positives or tracheal histopathology scores were not statistically significantly different ($P > 0.05$) between groups, using Fisher's exact test or Mann-Whitney's U test, respectively.

† birds drank potable water (Nil), or water containing plant essential oils or soluble yeast.

NA: Not Applicable

CI: Confidence Interval

This is consistent with findings from a previous study, where the same viral strain caused very severe ILT in both SPF and broiler chickens (15). Vaccination prevented these changes, demonstrating the high level of performance of eye-drop vaccination with the A20 vaccine strain, and consistent with previous studies that have reached a similar conclusion (26). Under these conditions, nutritional supplements had no measurable influence on the efficacy of vaccination, measured as the outcome of infection after challenge with virulent virus.

It is difficult to determine if vaccination protocols currently used in the field in Australia are effective against challenge with virulent virus. Antibodies are not protective against challenge (27-29) and there are no laboratory tests to measure cell-mediated immune responses that can be applied at large scale (30). Therefore, vaccination in the field, followed by challenge under experimental conditions is the only practical way to assess the efficacy of vaccination protocols currently in use. It is possible that the nutritional supplements may have an effect on vaccine efficacy under field vaccination conditions, such as using sub-optimal doses (for example during a vaccine shortage) or when vaccine is administered by the drinking-water route, which is common in the field. Drinking water vaccination has been associated with variable levels of vaccine uptake (31) and/or insufficient levels of protection against disease (32), with the additional complication that vaccination through bird-to-bird transmission does not necessarily lead to protection against challenge (27, 28, 33). Further studies would be required to investigate whether nutritional supplements have any effects on the efficacy of vaccination under these conditions.

It is interesting to note that chickens that received plant essential oils in the drinking water were equally protected against challenge infection as those that received no supplements or soluble yeast. Taken together with data from 4 days after vaccination, this finding indicates that an enhanced innate immune response and more efficient clearing of vaccine ILTV from infection sites did not interfere with the induction of an efficient adaptive immune response, or with resilience against ILTV re-infection. In addition to this, this work demonstrates that low levels of vaccine infection in the trachea, associated with eye-drop vaccination, are not an obstacle for the induction of an efficient local adaptive immune response in the trachea. This is despite very few lymphocytes located to the trachea at 4 days after eye-drop vaccination, consistent with the histopathology scores observed (Table 4). It is also interesting that tracheal swabs collected 7 days after challenge from unvaccinated chickens had significantly lower ($P = 0.029$, Student's T-test) ILTV Log_{10} GCN compared to conjunctival swabs, despite inoculation being directly into the trachea. This is in contrast to observations by Beltran *et al.* (2017), who determined much higher levels of virulent ILTV in the trachea than in conjunctiva in chickens inoculated via the intra-tracheal route (34). The different pattern observed here may be attributed to the ILTV strains used, or the age of inoculation.

Supplementation with essential oils was associated with increased weight gain by the end of the study. The body weight gain model was a reasonable fit to the data, with an acceptable distribution of the residuals (Supplementary Figure 2) and R^2: 0.586. The final weight gain parameter estimates from the weight model are described in Supplementary Table 4. The body weight gain (Figure 4) was positively associated with the initial bodyweight. An increase in weight gain was associated with vaccination, essential oil supplementation between 0-17 days of age, and male sex. A decrease in weight gain was associated with challenge. There was no apparent effect of supplementation with essential oils or soluble yeast between 0-35 days of age. This finding requires further investigation, as there are no previous data to better contextualise this observation.

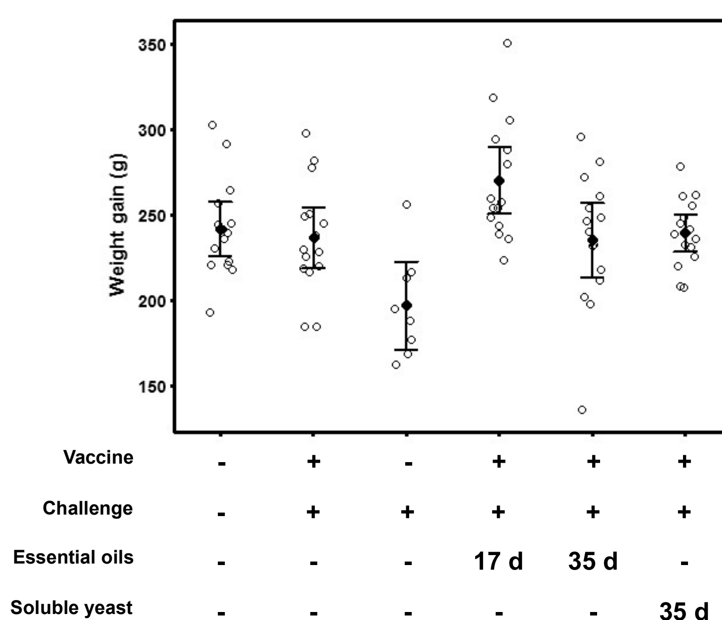

Figure 4. Individual, mean and 95% confidence interval weight gain between 6 and 35 days-of-age in specific-pathogen-free chickens after vaccination with A20 vaccine at 7 days of age and/or challenge with virulent ILTV at 28 days of age. Chickens received essential oils from hatch until 17 days, or essential oils or soluble yeast from hatch until the end of the study at 35 days of age.

Implications

Results from this project support the hypothesis that nutritional supplements, particularly plant essential oils, have positive effects on the safety characteristics of a commercial vaccine against ILT. More importantly, findings from this project demonstrate that plant essential oils have immune-modulatory effects that result in an enhanced innate immune response against infection with an attenuated ILTV vaccine virus. This enhanced anti-viral response does not interfere with the establishment of immunological memory and resilience in young chickens. It remains uncertain if the enhanced anti-viral

immune responses associated with the intake of plant essential oils are also effective for the control of virulent ILTV or other pathogens that infect young chickens.

Recommendations

Plant essential oils enhanced the anti-viral immunity against attenuated ILTV and prevented the reduction in weight gain associated with vaccination. There were no negative effects associated with the consumption of this product under the experimental conditions tested. Further studies using commercial chickens under field conditions would be required to determine if findings from this experimental investigation are transferrable to field settings. Nevertheless, the strong positive effects associated with plant essential oils here observed, warrant the use of this nutritional supplement in young chickens. These findings may be particularly relevant to ILTV vaccines with higher levels of residual virulence. This commercial product is readily available to the poultry industries in Australia.
Further studies to investigate whether these enhanced anti-viral effects are effective against virulent challenge with ILTV or other viruses would also be desirable.

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Media and Publications

A journal article describing the findings of this project is in preparation. Results from this project have been presented at the PoultryHub Australia Ideas Exchange conference in 2019. In addition to this, results from this project will be presented at the Australasian Veterinary Poultry Association Conference in May 2020 (online due to SARS-CoV2 pandemic). This work will also be presented at

the Avian Respiratory Viruses Symposium in Utrecht, the Netherlands, and the Marek's Disease and Avian Herpesvirus Symposium in Guelph, Canada (both re-scheduled due to SARS-CoV2 pandemic).

Intellectual Property Arising

None identified.

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Appendices

Supplementary Table 1. Parameter estimates and 95% confidence intervals (CI), obtained from the body weight gain at 4 days after vaccination model.

		95% CI	
Predictor	Estimate	Upper	Lower
Intercept	-2.6882	-16.7229	11.3467
Initial weight	0.6557	0.4075	0.9139
Vaccination	-12.7163	-18.3821	-7.0505
Essential oils	6.4000	1.8577	10.9423
Soluble yeast	2.7212	-3.1858	8.6283
Sex (male)	1.8894	-1.8639	5.6427

Supplementary Table 2. Parameter estimates and 95% confidence intervals (CI) for genome copy numbers of infectious laryngotracheitis virus as determined by qPCR in conjunctival, palatine cleft and tracheal swab samples collected from specific-pathogen-free chickens 4 days after inoculation with A20 vaccine.

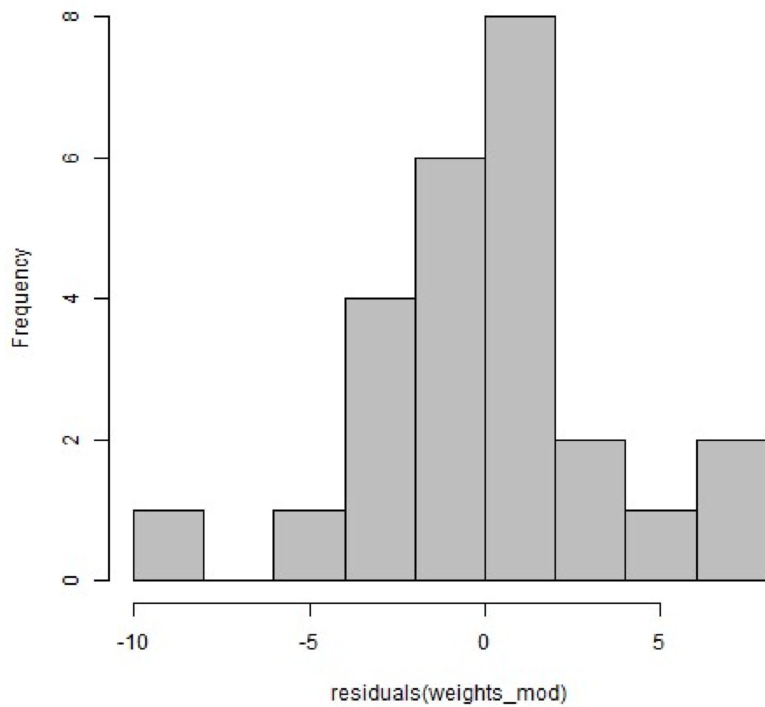
	Conjunctiva			Palatine Cleft			Trachea		
		95% CI			95% CI			95% CI	
Predictor	Estimate	Lower	Upper	Estimate	Lower	Upper	Estimate	Lower	Upper
Intercept	4.82	3.62	6.85	5.22	4.09	7.83	3.58	2.66	4.21
Vaccination	9.58	7.24	11.04	9.35	6.53	10.81	1.79	0.44	3.35
Essential oils	-1.59	-3.90	0.58	-3.22	-5.78	-0.88	-1.67	-3.07	-0.43
Soluble yeast	-0.18	-1.78	2.26	0.39	-1.21	3.32	-0.06	-1.49	1.66
Sex (male)	-0.68	-2.83	0.80	-1.21	-3.69	0.26	0.95	0.13	1.68

Supplementary Table 3. Parameter estimates and 95% confidence intervals (CI) for genome copy numbers of infectious laryngotracheitis virus (ILTV) as determined by qPCR in conjunctival, palatine cleft and tracheal swab samples collected from specific-pathogen-free chickens 7 days after challenge with a virulent ILTV strain.

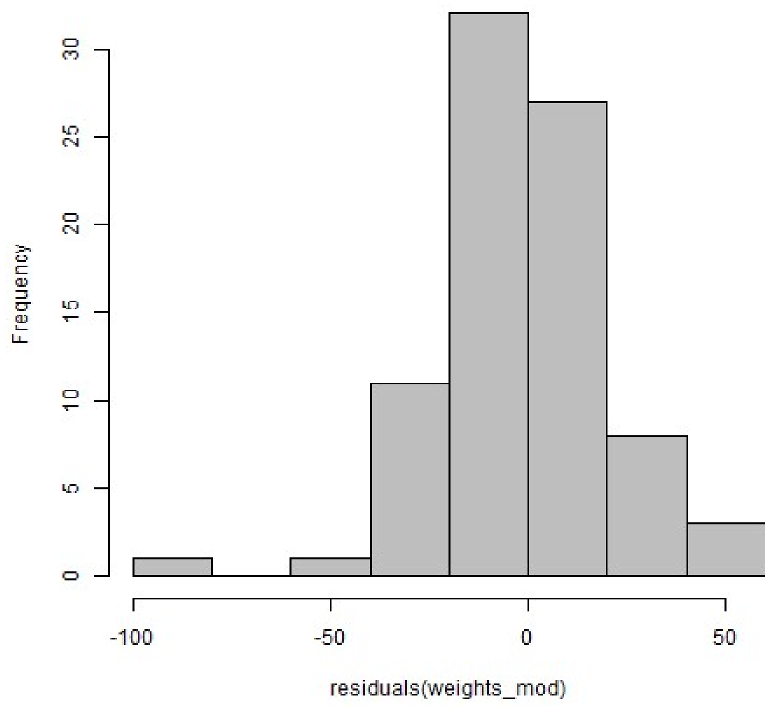
	Conjunctiva			Palatine Cleft			Trachea		
		95% CI			95% CI			95% CI	
Predictor	Estimate	Lower	Upper	Estimate	Lower	Upper	Estimate	Lower	Upper
Intercept	4.13	3.89	4.3	4.21	3.84	4.45	4.25	4.09	4.46
Vaccination	-5.74	-7.41	-4.05	-4.55	-6.56	-2.76	-2.62	-4.48	-1.29
Challenge	5.69	4.03	7.35	4.64	2.93	6.65	2.7	1.29	4.46
Essential oils 17 days	0.03	-0.15	0.18	0.27	-0.16	0.79	-0.06	-0.11	0.11
Essential oils 35 days	0.04	-0.12	0.24	1.16	0.39	1.86	-0.04	-0.11	0.13
Soluble yeast 35 days	0.17	0.08	0.26	0.67	0.08	1.25	-0.11	-0.12	0.13
Sex (male)	0.3	0.04	0.66	0.29	-0.24	0.74	-0.03	-0.24	0.36

Supplementary Table 4. Parameter estimates and 95% confidence intervals (CI), obtained from the final body weight gain model.

Predictor	Estimate	95% CI Upper	95% CI Lower
Intercept	98.4195	41.1481	155.6908
Initial weight	2.1401	1.1816	3.0985
Vaccination	29.5299	8.4922	50.5675
Challenge	-42.3582	-62.7018	-22.0144
Essential oils 17 days	36.8481	19.9677	53.7285
Essential oils 35 days	0.1796	-16.6550	17.0142
Soluble yeast 35 days	15.5610	-1.6810	32.8029
Sex (male)	33.3949	23.1394	43.6503



Supplementary Figure 1. Distribution of residuals for the body weight gain model applied between 1 day before and 4 days after vaccination.



Supplementary Figure 2. Distribution of residuals for the body weight gain model applied between 6 and 35 days of age.