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Feed supplementation with a mixture of C1 to C12 monoacylglycerides increases chicken resistance to *Salmonella* Enteritidis infection

Marcela Faldynova [a], Jiri Volf [a], Daniela Karasova [a], Hana Prikrylova [a], Alena Sebkova [a], Adela Mullerova [b], Renata Kucerova [b], Sylvie Skalickova [b], Ivan Rychlik [a,*]

[a] *Veterinary Research Institute, Hudcova 70, 62100, Brno, Czech Republic*
[b] *Department of Animal Nutrition and Forage Production, Mendel University in Brno, Faculty of Agrisciences, Zemedelska 1, 613 00 Brno, Czech Republic*

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ABSTRACT

Chickens represent one of the most important sources of animal protein for the human population. However, chickens also represent one of the most important reservoirs of *Salmonella* for humans. Measures to decrease the *Salmonella* incidence in chickens are therefore continuously sought. In this study, we tested feed supplementation with a mixture of C1 to C12 monoacylglycerides. At 0.7 and 1.5 kg per ton of feed, such supplementation significantly decreased *Salmonella* counts in the caecum but not in the liver. The chickens were infected on day 4 and the protective effect in the caecum was recorded on day 22 and 23 of life. Supplementation also decreased the inflammatory response of chickens to *Salmonella* infection determined by avidin, SAA, ExFABP, MMP7, IL1β, IL4I and MRP126 gene expression but did not affect immunoglobulin expression in the caecum. C1 to C12 monoacylglycerides can be used as a feed supplement which, if continuously provided in feed, decrease *Salmonella* counts in chickens just prior slaughter.

Introduction

Poultry and chickens in particular represent one of the most common sources of animal proteins for humans worldwide. However, poultry also represents a reservoir of zoonotic agents such as *Salmonella* or *Campylobacter*. Chicken colonisation by these two agents is usually without any clinical signs (Desmidt et al., 1997; Awad et al., 2018), which means the presence of these pathogens in poultry flocks can be overlooked and these are then transferred to humans via the food chain.

Multiple strategies have been tested to reduce chicken colonisation with *Salmonella* (Neelawala et al., 2024). Vaccination is used the most frequently but due to the short life of broilers, this can be used effectively only in egg layers or reproductive flocks (Pan et al., 2024). Probiotics or competitive exclusion products can be used as another alternative but single species probiotics usually based on *Lactobacillus, Enterococcus* or *Bacillus* species are not too effective against *Salmonella* (Khan and Chousalkar, 2020; Juricova et al., 2022; Olsen et al., 2022) and complex competitive exclusion products, though effective, raise concerns because of their undefined composition (Methner et al., 1997; Ferreira et al., 2003). Defined probiotics consisting of species different from *Lactobacillus* or *Bacillus* are only gradually introduced (Kubasova et al., 2021; Volf et al., 2024a) and phages have not proved their efficacy in real conditions (Agape et al., 2024). However, there is great potential for the modification of feed composition by supplementation with components suppressing pathogen multiplication in the chicken intestinal tract.

Different feed supplements have been tested in chickens including plant extracts or short- and medium-chain fatty acids or their esters with glycerol (monoacyl glycerides) (Van Immerseel et al., 2005; Varmuzova et al., 2015; Jackman et al., 2022). The latter supplements are similar in the mode of action (Jackman et al., 2022). Both fatty acid and monoacyl glycerides pass freely through the bacterial outer and cytoplasmic membrane. Inside the cell, fatty acids in the cytoplasm dissociate into the negatively charged residue of the acid and proton H^+. The release of H^+ results in acidification of the cytoplasm and interference with intracellular metabolism. The same action is expected for monoacyl glycerides, which, following the passage through the membranes, are cleaved by intracellular host enzymes and free fatty acid is released. Since glycerol feed supplementation provided conflicting results (Delgado et al., 2014; Ozdogan et al., 2014) and the comparison

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* Corresponding author.
E-mail address: rychlik@vri.cz (I. Rychlik).

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between free capric acid and capric acid monoglyceride showed identical antibacterial effect *in vitro* (Thormar et al., 2006), fatty acids are central for antibacterial activity. Indeed, positive results with fatty acid supplementation have been repeatedly reported by different authors although conclusions of different studies may differ in the range of efficacy of particular fatty acids for specific target microorganisms (Van Immerseel et al., 2004a; Van Immerseel et al., 2004b; Thormar et al., 2006). Unfortunately, if provided orally, fatty acids are resorbed by the host in the small intestine and do not efficiently reach the distal parts of the intestinal tract. This has been addressed by fatty acid encapsulation (Van Immerseel et al., 2005; Boyen et al., 2008; Onrust et al., 2020) or glycerol esterification resulting in monoacyl glycerides with delayed release of free fatty acids in the intestinal tract (Gomez-Osorio et al., 2021).

Monoacylglycerides have been shown to inactivate both Gram-negative *Escherichia coli* and Gram-positive *Listeria monocytogenes* (Wang and Johnson, 1992; Wang et al., 2018). Due to their interactions with membranes, they are effective also against enveloped viruses (Hariastuti et al., 2010). Anti-*Salmonella* effects of monoacyl glycerides have been reported as well (Thormar et al., 2006; Chen et al., 2021). However, these studies used monoacyl glycerides for direct *Salmonella* inactivation, either in *in vitro* experiments (Thormar et al., 2006) or for carcass surface disinfection (Chen et al., 2021). The anti-*Salmonella* effect by feed supplementation has not been tested although a positive effect of monoacylglycerides on the alleviation of clinical signs of necrotic enteritis caused by *Clostridium perfringens* has been reported (Gharib-Naseri et al., 2021; Daneshmand et al., 2023).

In this study, we therefore tested feed supplementation with a unique mixture of short and medium carbon chain (C1 to C12) monoacylglycerides whether i) it will affect *Salmonella* colonisation in chickens and ii) it will decrease the inflammatory response to *Salmonella* infection. For both tested hypotheses, we recorded significant positive outputs although the effect was not numerically extensive.

Material and methods

Ethics statement

The handling of animals in the study was performed in accordance with current Czech legislation (Animal Protection and Welfare Act No. 246/1992 Coll. of the government of the Czech Republic). The specific experiments were approved by the Ethics Committee of the Veterinary Research Institute and the Committee for Animal Welfare of the Ministry of Agriculture of the Czech Republic (permit number MZe2405 approved on March 5, 2023).

Experimental design

The whole study was performed in 2 independent experiments. In both experiments, chickens in the control group were provided basal feed and two experimental groups were fed the basal feed supplemented with either 0.7 or 1.5 kg of C1-C12 monoacylglycerides per 1 000 kg of feed (Fortibac, Addicoo, Czech Republic). The product consisted of a mixture of equal amount of monoformin (C1), monopropionin (C3), monobutyrin (C4), monocaprylin (C8), monocaprin (C10) and monolaurin (C12). Mixture of monoacylglycerides was mixed with SiO_2 used as a vehiculum in 65:35 weight/weight ratio. Supplemented feed was provided to the chickens throughout the whole experiment. There were 24 newly hatched male ISA Brown egg laying chickens in each of the 3 groups in the beginning of the first experiment, *i.e.* altogether 72 chickens. The same numbers of chickens per group were used also in the second experiment. However, since an additional group of non-infected chickens fed a basal diet was included, there were 4 groups and altogether 96 chickens in the repeated experiment. In addition, 3 chicks in each experiment were sacrificed on day 1 upon arrival to animal house to test for *Salmonella* negativity and all indeed tested negative.

On day 4 life, 6 chickens in each group were orally inoculated with 10^7 CFU/mL of *Salmonella* Enteritidis 147 (*S*. Enteritidis) spontaneously resistant to nalidixic acid in 0.1 mL volume (except for the control group of non-infected chickens in the second experiment). Infected chicks were identified by leg rings and acted as seeder birds for the remaining contact chicks in each group. Six contact chicks from each group were sacrificed 4, 11 and 18 days post infection. Seeder birds were sacrificed at the end of each experiment at 19 days post infection.

Sample collection

Birds were euthanised by carbon dioxide inhalation followed by cervical dislocation. During dissection, approx. 0.5 g of caecal content and 0.5 g of liver tissue were removed to determine *Salmonella* counts. In the second experiment, small pieces of the caecum were placed in RNALater and stored at -20 °C prior to RNA purification. Caecum was selected as major site of *Salmonella* colonisation in chickens. In addition, blood samples were collected immediately after decapitation. Following blood coagulation at 4 °C for 16 hours, the samples were centrifuged at 2000x g and sera were collected and stored at -20 °C.

Salmonella culture

Caecal contents and liver tissue samples were homogenised in 5 mL peptone water, tenfold serially diluted and plated on Xylose Lysine Deoxycholate agar supplemented with nalidixic acid. *S*. Enteritidis colonies were counted after 48-hour incubation at 37 °C. All peptone water homogenates were incubated at 37 °C for this period as well. In the case of no *Salmonella* colonies after direct plating, peptone water homogenates were processed according to ISO 6579 protocol for qualitative *Salmonella* detection. *S*. Enteritidis counts were logarithmically transformed and samples positive only after ISO protocol were assigned a value of 1. *Salmonella* negative samples were given a value of 0.

ELISA quantification of serum amyloid A protein (SAA) in blood serum

Blood serum samples were collected after decapitation during dissections and stored at -20 °C until used for the quantification of SAA by ELISA (Chicken SAA (serum amyloid A) ELISA Kit 96T, Fine Test). Prior to ELISA assay, serum samples were diluted 500 times in PBS and each sample was analysed in duplicate. SAA standard from the kit was used for the production of a calibration curve and SAA concentration in μg/mL was calculated for each sample.

Quantitative reverse transcribed real time PCR (qPCR)

Samples of chicken caecal tissues (50–100 mg) were homogenised in TRI Reagent and RNA was recovered from the upper water phase following the instructions of the manufacturer (MRC). mRNA was immediately reverse transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen) and oligo (dT) primers. cDNA was diluted 10 times with sterile water prior to real-time PCR. PCR was performed in 3 μL volumes in 384-well microplates using QuantiTect SYBR Green PCR Master Mix (QIAGEN) and a NanoDrop pipetting station (Innovadyne) for PCR mix dispensing. Expression of 8 genes known as part of the chicken response to *Salmonella* infection was determined by qPCR. These included avidin (AVD), serum amyloid A (SAA), extracellular fatty acid binding protein (ExFABP), matrix methalloproteinase 7 (MMP7), interleukin 1β (IL1β), IL-4 induced 1 protein (IL4I), macrophage migration inhibitory factor (MIF)-related protein 126 (MRP126) and light chain immunoglobulin (Ig λ) (Matulova et al., 2013; Volf et al., 2017; Elsheimer-Matulova et al., 2020; Volf et al., 2024b). The Ct values of the genes of interest were normalised (ΔCt) to a geometric mean of Ct value of 3 reference genes, TBP1, HMBS and ADA, and the relative expression of each gene of interest was calculated as $2^{\Delta Ct}$. All the primers are listed in Table 1.

Table 1
List of primers used in this study.

Target gene	Forward primer	Reverse primer
AVD	CCTTTGGCTTCACTGTCAAT	GCGAGTGAAGATGTTGATGC
SAA	TAGTTTGCCTCACGCATGTC	GCTTCGTGTTGCTCTCCATT
EXFABP	CTTGCACATGATGAGGCTCT	CAGCATTCATCAGCCATCC
MMP7	GATGATGCAATTAGAAGGGCTTT	CCACCTCTTCCATCAAAAGGATA
MRP126	TGAAGCTCTTGATTGAGAAGCA	CGAGATCCTTGAAGATTTGGTC
IL1b	GAAGTGCTTCGTGCTGGAGT	ACTGGCATCTGCCCAGTTC
IL4I1	GGAGAAGGACTGGTATGTGGAG	GCTTCAGGTCAAACTGCCTTAT
IgL	TGCAATGTGAGGACAGTGGT	GAGGAGTCAACAGGCAGAGG
TBP1	TAGCCCGATGATGCCGTAT	GTTCCCTGTGTCGCTTGC
HMBS	GGCTGGGAGAATCGCATAGG	TCCTGCAGGGCAGATACCAT
ADA	TATCAACACCGATGACCCCC	GCTGGACTGAGCTGCATTGA

Statistics

Salmonella counts were evaluated separately for each experiment as well as combined in a single dataset. *Salmonella* counts in orally infected seeder birds were treated separately from the contact chickens. t-test was used to evaluate difference in *S*. Enteritidis counts in the caecum and liver of control and experimental chickens. t-test was used also for the comparison of chicken gene expression and SAA levels in blood serum.

Results

Monoacylglyceride supplementation and chicken resistance to Salmonella

Feed supplementation with a mixture of monoacylglycerides affected chicken resistance to *Salmonella* colonisation. Significantly higher *Salmonella* counts were recorded in the caeca of chickens fed diet with 1.5 kg monoacylglycerides per metric ton than that in the control chickens in the first experiment on day 15. On the other hand, significantly lower *Salmonella* counts were recorded in the seeder birds fed a diet supplemented with 0.7 kg monoacylglycerides per metric ton on day 23 (Fig. 1A).

In the repeated experiment, significantly lower *Salmonella* colonisation was recorded in the caeca of contact chickens from both experimental groups on day 22. In addition, significantly lower *Salmonella* colonisation was recorded also in the caeca of seeder chickens from both experimental groups on day 23 (Fig. 1B).

When data from both experiments were combined, a significantly lower level of *Salmonella* colonisation was recorded in the caeca of contact and seeder chickens from both experimental groups on days 22 and 23. Unlike caecal colonisation, the supplementation did not affect systemic spread of *Salmonella* since there were no significant differences in *S*. Enteritidis counts in the liver among different groups (Fig. 1). Monoacylglycerides therefore acted against *Salmonella* only in the intestinal tract and when *Salmonella* entered the circulation and systemic sites, *Salmonella* was protected against their activity.

Comparison of gene expression in infected and non-infected chickens

In the second experiment, caecal tissue samples were collected to determine inflammatory response to *S*. Enteritidis infection. In addition, acute response was characterised also by ELISA quantifying SAA in blood sera. Key differences in comparison to the control non-infected chickens were recorded on day 15 of life, *i.e.* 11 days post infection, when the highest inflammatory response was recorded in the chickens fed with a basal diet. An intermediate inflammatory response to *Salmonella* infection was detected in the chickens fed a diet with high monoacylglyceride supplementation and the lowest response among infected chickens was recorded in the chickens provided feed with low monoacylglyceride supplementation (Fig. 2). Specifically, significantly higher expression of avidin, SAA, IL1β, ExFABP and MRP126 was recorded in *Salmonella* infected chickens fed a basal diet than in the non-infected chickens on day 15. Chickens provided feed with a high amount of monoacylglycerides also mounted an inflammatory response since the expression of avidin, SAA, IL1β, ExFABP and MMP7 was significantly higher than in the non-infected chickens. Despite significant inductions, the increase in the expression in chickens fed a diet with high monoacylglyceride supplementation was numerically lower than that in chickens fed a basal diet. Chickens fed a high monoacylglyceride diet also exhibited significantly higher expression of IL4I on day 8 in comparison to those fed a basal diet.

IL1β was the only tested marker which was significantly more expressed in the chickens provided feed with low monoacylglyceride supplementation in comparison to the non-infected controls on day 15. Chickens in this group exhibited also a significantly higher level of avidin and MRP126 expression as early as on day 8.

Gene expression of Ig λ was not affected by *Salmonella* infection or feed supplementation.

Comparison of gene expression in infected chickens fed a basal and supplemented diet

A lower inflammatory response to *Salmonella* infection and therefore protective effect of tested supplements was confirmed also by the comparison of gene expression in *Salmonella* infected chickens fed basal and supplemented diet. Avidin, ExFABP and MMP7 were significantly less expressed in the infected chickens fed a diet with high monoacylglyceride and avidin, SAA and ExFABP were significantly less expressed in chickens fed a diet with low monoacylglycerides supplementation on day 15 in comparison to chickens fed a basal, non-supplemented diet (Fig. 2). All this collectively showed that feed supplementation reduced the inflammatory response to *Salmonella* infection although not to the levels recorded in the non-infected chickens.

ELISA quantification of SAA in blood serum

SAA was determined also in blood sera by ELISA. None of the comparisons in SAA levels between infected and non-infected chickens reached statistical significance (Fig. 2). However, when comparing results from real-time PCR in the caecum and ELISA in blood serum, the highest expression SAA levels were detected by both protocols in the infected chickens fed a basal diet on day 15 and in infected chickens fed the diet with low monoacylglyceride supplementation on day 22.

Discussion

Salmonellosis still belongs among the most common gastrointestinal disorders in humans. Since chickens represent one of the most common reservoirs of different *Salmonella* serovars, measures on how to improve gut health and decrease *Salmonella* prevalence in poultry are continuously sought. Of extra value are the solutions which are simple and cheap to introduce, such as the use of different feed additives. Short and medium fatty acids and their monoesters with glycerol belong among such additives with a proven effect on growth performance, intestinal morphology, meat and egg quality (Fortuoso et al., 2019; Feng et al., 2021a; Feng et al., 2021b; Chen et al., 2024; Kerr et al., 2024; Wang et al., 2024) as well as activity against *Campylobacter, Salmonella* or spoilage microbiota *in vitro*, or *Clostridium perfringens* and *Campylobacter in vivo* (Hilmarsson et al., 2006; Thormar et al., 2006; Namkung et al., 2011; Thormar et al., 2011; Gomez-Osorio et al., 2021; Chen et al., 2021; John et al., 2024)). Since the effect of monoacylglycerides against *C. perfringens* or *Campylobacter in vivo* was rather moderate, we expected the same in the case of *Salmonella*. This is why the seeder bird model, in which the contact chickens are subjected to lower infection pressure than the seeder birds directly inoculated with high doses of *Salmonella*, was used. Due to this model, rather low *Salmonella* counts were recorded 4 days post infection and maximal colonisation occurred later, at 11

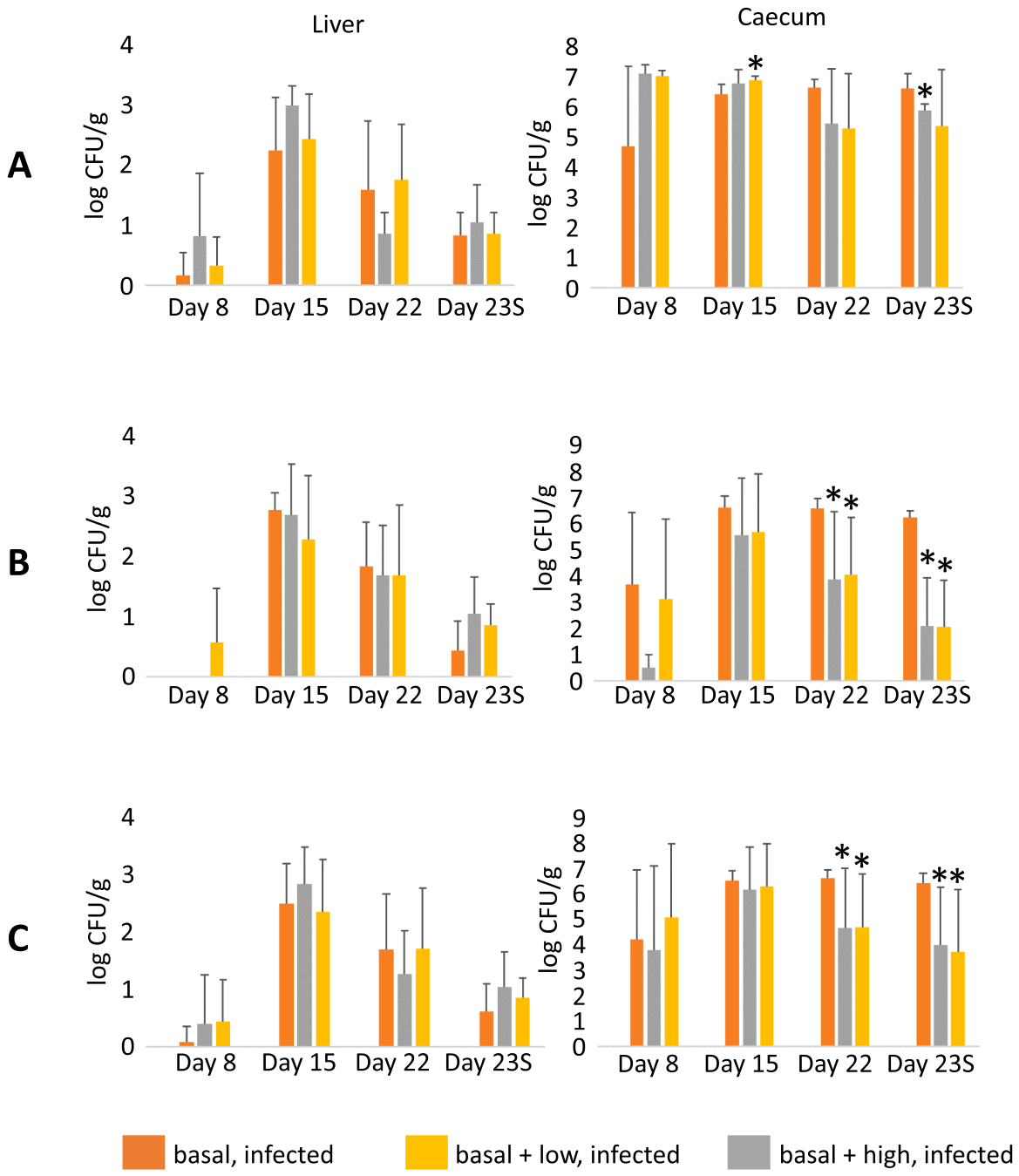

Fig. 1. *Salmonella* counts in chickens fed differentially supplemented feed. Prolonged feed supplementation with monoacylglycerides increased chicken resistance to *Salmonella* colonisation. Significantly lower *Salmonella* counts were recorded in the caecum on day 23 in the seeder birds in the first experiment (Panel A). Significantly lower *Salmonella* counts were recorded on days 22 and 23S ("S" stands for Seeders) in the second (Panel B) and also when data from both experiments were combined (Panel C). Feed supplementation with monoacylglycerides did not affect *S.* Enteritidis dissemination and persistence in the liver (Panels A-C). * - significantly different ($P < 0.05$) from *Salmonella* counts in the caeca of chickens fed a basal, non-supplemented diet.

days post infection, although peak in *Salmonella* caecum colonisation is recorded usually 4 days post infection (Beal et al., 2004; Matulova et al., 2013).

C1 to C12 monoacylglycerides did not increase chicken resistance to infection immediately. Instead, in both experiments, continuous in-feed administration resulted in lower *Salmonella* counts 18 and 19 days post infection in both experiments, even though the difference did not reach statistical significance in the first experiment due to high chicken to chicken variation. In addition, there was no effect of feed supplementation on *Salmonella* liver colonisation, likely due to the fact that monoacylglycerides do not enter the circulation to affect *Salmonella* persistence in the liver. The fact that *Salmonella* counts decreased at a later time during fattening means that such treatment may effectively decrease chicken colonisation at the time of slaughter, similar to conclusions of Fernandez-Rubio et al, though they used sodium butyrate supplementation and not acylglycerides (Fernandez-Rubio et al., 2009). Beneficial effect of monoacylglyceride supplementation was observed also in laying hens at the late stage of egg production (Feng et al., 2021a; Wang et al., 2024).

Monoacylglycerides are known also for their anti-inflammatory effect (Kong et al., 2021; Chen et al., 2024; Kong et al., 2024). In agreement, we recorded a lower inflammatory response in the chickens fed a

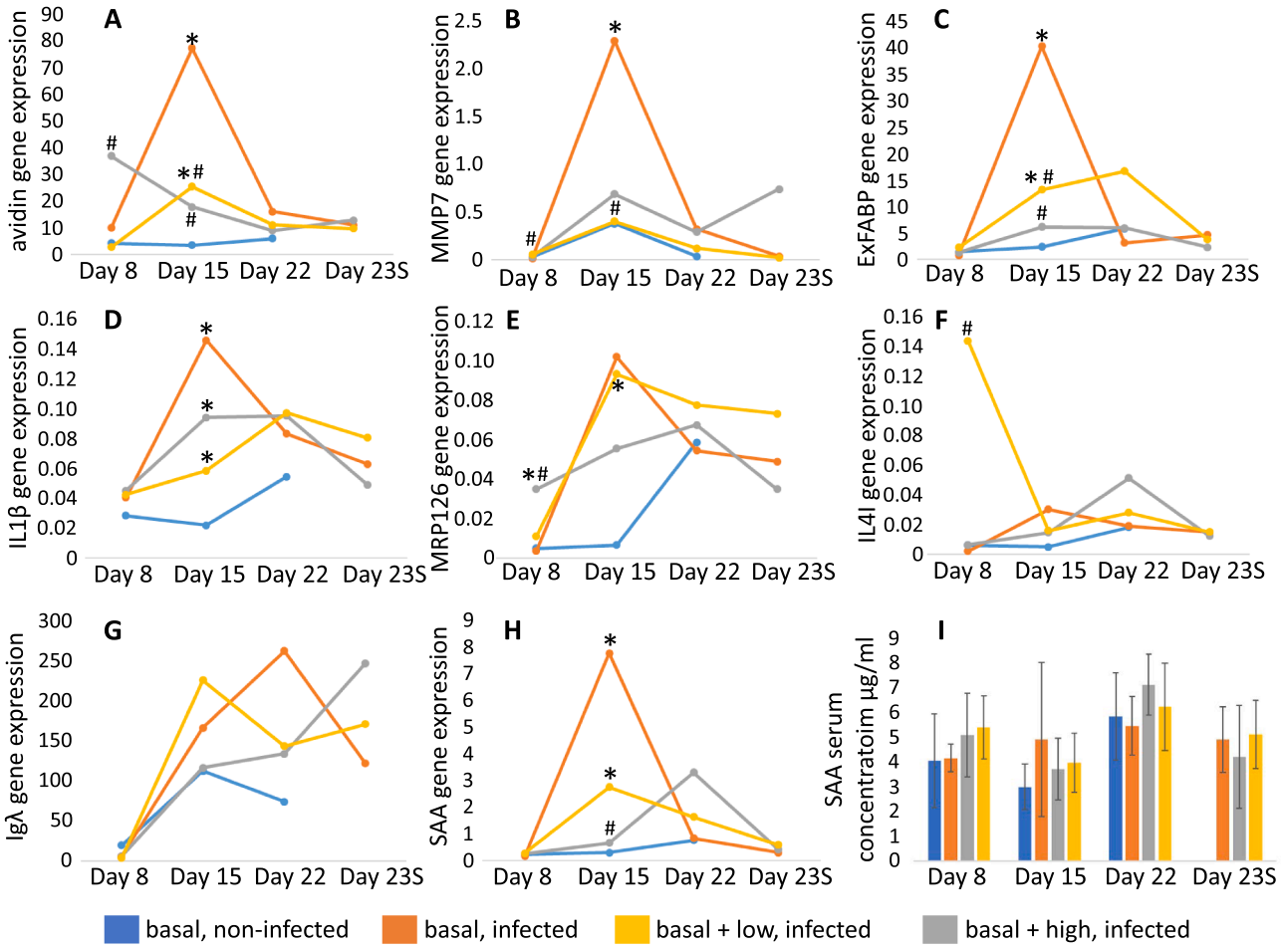

Fig. 2. Gene expression of selected inflammatory marker genes in the chicken caecum following *Salmonella* infection. *Salmonella* infection resulted in the induction of an inflammatory response on day 15 of life, *i.e.* 11 days post infection. A rather delayed onset of the inflammatory response was influenced by the established model of seeders and contact chickens. The highest inflammatory response was always recorded in the chickens fed a basal diet while supplementation of feed with monoacylglycerides resulted in an intermediate expression, in between of non-infected and infected, basal feed fed chickens (Panels A-H). Panel I - ELISA quantification of SAA in blood serum of *Salmonella* infected chickens fed differentially supplemented feed did not result in significant differences among individual groups of chickens, though numerically the highest levels of SAA on day 15 were recorded in the chickens fed a basal diet by both real-time PCR and ELISA (compare orange line for SAA in panel H and orange column on day 15 in panel I) and similarly, the numerically highest levels of SAA on day 22 were recorded in the chickens fed a diet with low monoacylglyceride supplementation (compare grey line for SAA and grey column on day 22). Six chickens in each group were analysed at each time point.

diet supplemented with monoacylglycerides but we did not observe any effect on immunoglobulin expression though chickens respond to *Salmonella* and normal microbiota by the induction of immunoglobulins (Matulova et al., 2013; Volf et al., 2017; Volf et al., 2024b). It is difficult to make conclusions about direct anti-inflammatory effect of the supplementation since the more likely explanation would be that the tested supplements acted against *Salmonella* and lower *Salmonella* counts caused a lower inflammatory response. However, even if this is the case, lower inflammation may keep resorptive functions of the gut fully preserved (Varmuzova et al., 2014).

The absence of an Ig response to *Salmonella* infection was likely caused by the model used. Contact chicks were infected with low doses of *Salmonella* at around day 10 of life and the older the chicks are, the more resistant to *Salmonella* infection they are (Beal et al., 2004; Crhanova et al., 2011). Ig response to commensal microbiota colonisation can be seen only in comparison of colonised and germ-free chickens (Volf et al., 2017) and differences in Ig expression among chickens colonised by complex microbiota of different composition is likely below the discrimination power of real-time PCR.

Conclusions

In this study, we have shown that feed supplementation with a mixture of C1 to C12 monoacylglycerides significantly reduced *Salmonella* counts and corresponding inflammatory response in the caecum. The supplementation decreased *Salmonella* counts at around 3 weeks of age, which might be relevant for the presence of *Salmonella* in the chickens 10-14 days later at the time of slaughter. Monoacylglycerides did not protect chickens against *Salmonella* spread into systemic sites such as the liver and also did not affect natural level of immunoglobulin gene transcription in the caecum. Both tested supplementation doses, *i.e.* 0.7 as well as 1.5 kg per ton of feed performed similarly. Considering other beneficial effects of monoacylglycerides (Thormar et al., 2006; Kong et al., 2021; Jackman et al., 2022; Daneshmand et al., 2023), these can be used as feed supplement also in the flocks facing issues with *Salmonella*.

Disclosures

The authors declare that they have no competing interests.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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