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AVIAN INFLUENZA VIRUS TRANSMISSION IS SUPPRESSED IN CHICKENS FED *LACTOBACILLUS PARACASEI* EXPRESSING THE 3D8 SINGLE-CHAIN VARIABLE FRAGMENT PROTEIN

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The 3D8 single-chain variable fragment (scFv) is a mini-antibody sequence with independent nuclease activity that shows antiviral effects against all types of viruses in chickens and mice. In this study, chickens were treated daily with an oral dose of 10^9 CFU *Lactobacillus paracasei* (*L. paracasei*) expressing either a secreted or anchored 3D8 scFv for three weeks. After *L. paracasei* administration, the chickens were challenged with avian influenza virus (AIV). From each experimental group, three chickens were directly infected with 100 μL of $10^{7.5}$ EID_{50}/mL H9N2 AIV and seven chickens were indirectly challenged through contact transmission. Oropharyngeal and cloacal swab samples were collected at 3, 5, 7, and 9 days post-inoculation (dpi) from AIV-challenged chickens, AIV Shedding titres were measured by quantitative real-time PCR. Contact transmission in the chickens that were fed 3D8 scFv-secreting *L. paracasei* showed a significant reduction in viral shedding when compared with other groups. These results suggest that *L. paracasei* secreting 3D8 provides a basis for the development of ingestible antiviral probiotics with activity against AIV.

Key words: 3D8 scFv, avian influenza virus, *Lactobacillus paracasei*, oral administration

Probiotics are live microorganisms that can regulate fat metabolism by affecting genes that encode key enzymes (Zhu et al., 2015; Kang et al., 2016). Probiotics can effectively stimulate the immune system (Dec et al., 2015; Patel et al., 2015). Among alternatives to antibiotics in the poultry industry, the use of *Lactobacillus* as an immune stimulant to enhance nonspecific host defence mechanisms or as an antimicrobial to inhibit bacterial growth has been reported

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(Meimandipour et al., 2010; Wang et al., 2013; Ritzi et al., 2014; Kang et al., 2016). The previously described probiotics include therapeutic agents such as ingestible antiviral probiotics that are active against gastrointestinal viral infection (Hoang et al., 2015). Park et al. (2018) have developed a strain of *Escherichia coli* (*E. coli*) producing nucleic acid-hydrolysing codon optimised 3D8 single-chain variable fragment (3D8 scFv) for use as a feed additive to prevent norovirus infection (Park et al., 2018). *Lactobacillus paracasei* (*L. paracasei*) was shown to express a heavy-chain antibody fragment against rotavirus (Pant et al., 2006).

The 3D8 scFv is an anti-nucleic acid antibody that can bind and hydrolyse nucleic acids without sequence specificity (Kim et al., 2006). 3D8 scFv penetrates cells via the caveolae-lipid raft pathway (Hoffmann et al., 2001). Previous data confirmed that the 3D8 scFv protein suppresses avian influenza virus transmission in transgenic chickens (Byun et al., 2017). In addition, infectious bronchitis virus transmission is suppressed in transgenic chickens expressing the 3D8 scFv protein (Lee et al., 2019). Hence, 3D8 scFv antiviral activity against various viral infections is broad spectrum, and our study primarily aimed to investigate the prospect of using *L. paracasei* as a delivery system for 3D8 scFv to suppress avian influenza virus in chickens. We confirmed virus shedding in oropharyngeal and cloacal swab samples by quantitative real-time PCR in direct and contact transmission groups. Our findings showed that *L. paracasei* expressing 3D8 scFv could be developed for use as a feed additive to control AIV transmission.

Materials and methods

Ethics statement

The challenge study with a live virus was conducted in a biosafety level 2 facility under the supervision of the Institutional Animal Care and Use Committee (IACUC) (2015-111) of Konkuk University, South Korea.

Experimental design, animals and their diets

Forty conventional healthy specific-pathogen-free (SPF) chickens (three-week-old) were used in the experiment which lasted for 35 days. Chickens were allocated to four experimental groups and orally administered 10^9 colony-forming units (CFUs) (per chicken/day) of L. paracasei secreting 3D8 scFv (Group 1, G1); *L. paracasei* anchored 3D8 scFv (Group, G2); *L. paracasei* (Group 3, G3); control [Group 4 (G4) – phosphate-buffered saline (PBS)] using a syringe and blunt-end catheter. Every chicken was individually identified by using tags, and they received water and feed supplied *ad libitum*. The basal diets were formulated to exceed the nutritional requirements of chickens according to the NRC (1994) recommendations for experimental chickens.

In vivo *AIV infection and transmission studies*

After *L. paracasei* oral administration, we initiated AIV challenge in the treatment groups. Three chickens per group were intranasally challenged with 100 µL of $10^{7.5}$ EID_{50}/mL H9N2 virus (A/Korean native chicken/Korea/K040110/2010). The remaining seven chickens in each group were co-housed in the same containment isolator for 6 h post-inoculation. To assess viral shedding, oropharyngeal and cloacal swab samples were collected at 3, 5, 7, and 9 days post-inoculation (dpi) and suspended in 1 mL of PBS. Suspensions (200 µl) were used for RNA extraction using an RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The virus shedding values were measured according to Lee et al. (2011). The virus stock was propagated in the allantoic cavity of 10-day-old chicken embryos (n = 4) at 37 °C for 72 h according to Spackman et al. (2003). The allantoic fluids were harvested, aliquoted, and stored at −70 °C until use. The virus titre in the allantoic fluid was determined based on the 50% egg infective dose (EID_{50}). The EID_{50} values were measured according to Lee et al. (2011). Viral RNA was extracted from swab samples using the RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed under standard conditions using influenza-specific primers according to Hoffmann et al. (2001) (Fig. 1C). Amplified products of the expected size were purified with QIAquick PCR purification kits (QIAGEN), and nucleotide sequencing was performed (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions (Lee et al., 2011).

Construction of recombinant L. paracasei *pSLP-LDH expressing 3D8 scFv*

The pSLP111.3 expression vector for *Lactobacillus,* provided by Dr. Jos Seegers, was improved to replace the xylose-inducible promoter with a lactate dehydrogenate (LDH) constitutive promoter (Oozeer et al., 2005). To induce 3D8 scFv protein expression, codon-optimised 3D8 scFv was cloned into pSLP-LDH that had been slightly modified from the original vector as previously described (Hoang et al., 2015). 3D8 scFv *L. paracasei* was anaerobically cultured in de Man, Rogosa and Sharpe medium (Difco Laboratories, Detroit, MI) at 37 °C for 24 h. To construct the secreted form of 3D8 scFv, the 3D8 scFv gene fused with a protein A tag was inserted downstream of the SlpA secretion signal using the NcoI and AscI restriction enzyme sites. In addition, a stop codon (TAA) was added after the protein A tag to prevent fusion of the 3D8 scFv with the cell wall-anchoring domain of PrtP (anchor sequence). A cell wall-anchored form of 3D8 scFv was generated by inserting the 3D8 scFv gene using the NcoI and AscI restriction enzyme sites upstream of the anchor sequence according to Hoang et al. (2015) (Fig. 1A).

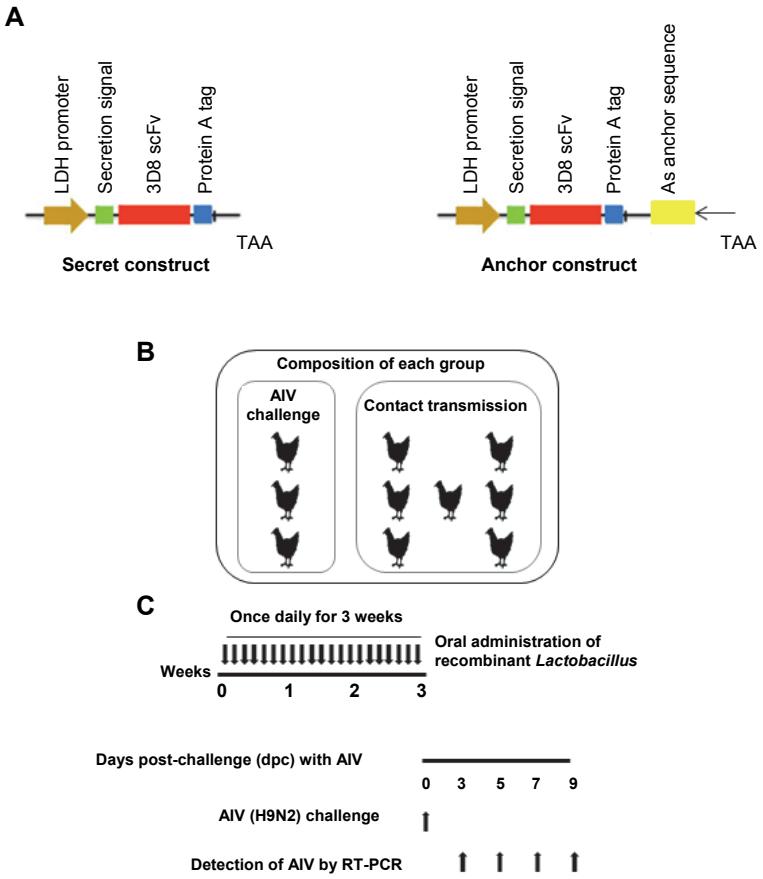

Fig. 1. Comparison of 3D8 scFv expression between *L. paracasei* expressing codon-optimised and the original sequence of 3D8 scFv (A): Expression cassette designed to express 3D8 scFv as a secreted form (left) or anchored on the *L. paracasei* cell wall (right). Chickens were assigned to four groups, with 10 chickens per group. Three chickens were directly infected with 100 µL of $10^{7.5}$ EID_{50}/mL H9N2 avian influenza virus (AIV challenge), and seven chickens were indirectly challenged (contact transmission) (B). Oropharyngeal and cloacal swabs samples were collected at 3, 5, 7, and 9 d post-inoculation (dpi) from AIV-challenged chickens and contact transmission chickens (C)

Statistical analysis

Data analyses were conducted using GraphPad Prism statistical software (GraphPad Software). Challenge virus results were measured in oropharyngeal and cloacal swabs using RT-PCR, and the data were compared using one-way ANOVA and Tukey's tests. P values of less than 0.05 were considered statistically significant. Error bars denote standard deviation (SD).

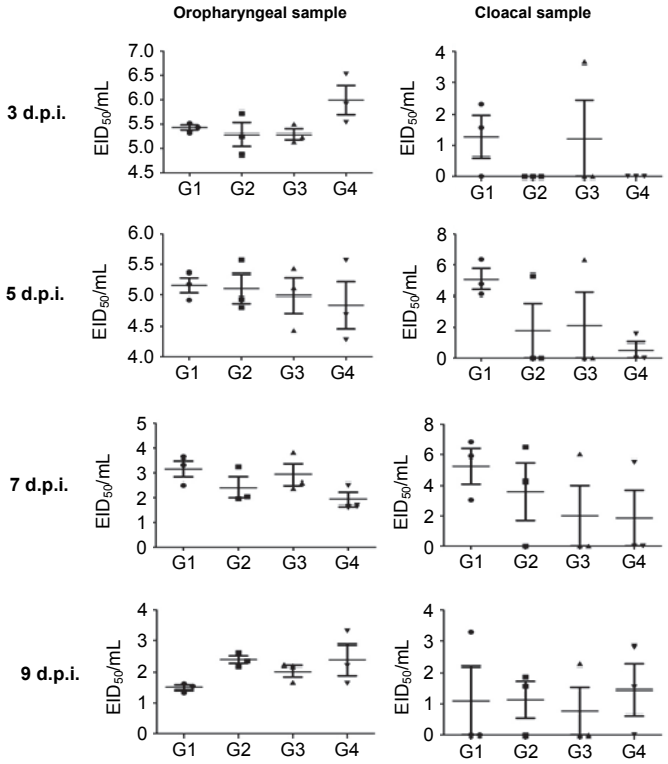

Fig. 2. Inhibition of AIV shedding levels after the oral administration of *L. paracasei* expressing the 3D8 in chicken oropharyngeal and cloacal swabs of the directly challenged group. Three-week-old specific-pathogen-free (SPF) chickens were housed in each containment cage in a biosafety level 2 (BSL2) animal facility. Three chickens were intranasally challenged with 100 μL of $10^{7.5}$ EID_{50}/mL H9N2 virus. Oropharyngeal and cloacal swab samples were harvested at 3, 5, 7 and 9 days post-inoculation (dpi) from the challenged chickens and the viral RNA was quantified by real-time RT-PCR. Mean viral shedding titres were calculated. Data bars represent the mean ± standard error

Results and discussion

In a previous study, our research team constructed the pSLp111.3 vector for expression in *Lactobacillus* provided by Dr. Jos Seegers (Falcobio, Netherlands). To induce 3D8 scFv protein expression, codon-optimised 3D8 scFv was cloned into pSLP-LDH that was slightly modified from the original vector as previously described (Hoang et al., 2015). Over the last two decades, the application of probiotics in the antiviral field has consisted primarily of anchoring viral antigens on the cell wall of probiotics in order to trigger an immune response (Seegers, 2002; Lee et al., 2006) or using probiotic-secreting proteins that act on cell surfaces to interrupt fusion between cellular and viral membranes (Rao et al.,

2005; Vangelista et al., 2010). In a different approach, in the present study, chickens were fed 10^9 colony-forming units (CFU) of *L. paracasei* secreting 3D8 scFv or *L. paracasei* expressing cell wall-anchored 3D8 scFv using oral administration. We investigated the effect of oral administration of 3D8 scFv-secreting *L. paracasei* using the experimental procedure shown in Fig. 1A. Each group included 10 chickens (H9N2 challenge: 3; contact transmission: 7) aged 3 weeks (Fig. 1B). After *L. paracasei* administration, the chickens were challenged with avian influenza virus. After challenge, individual chickens were monitored for 9 days. Oropharyngeal and cloacal swabs were collected at 3, 5, 7, and 9 dpi. The swabs were analysed by RT-PCR to detect AIV H9N2 (Fig. 1C).

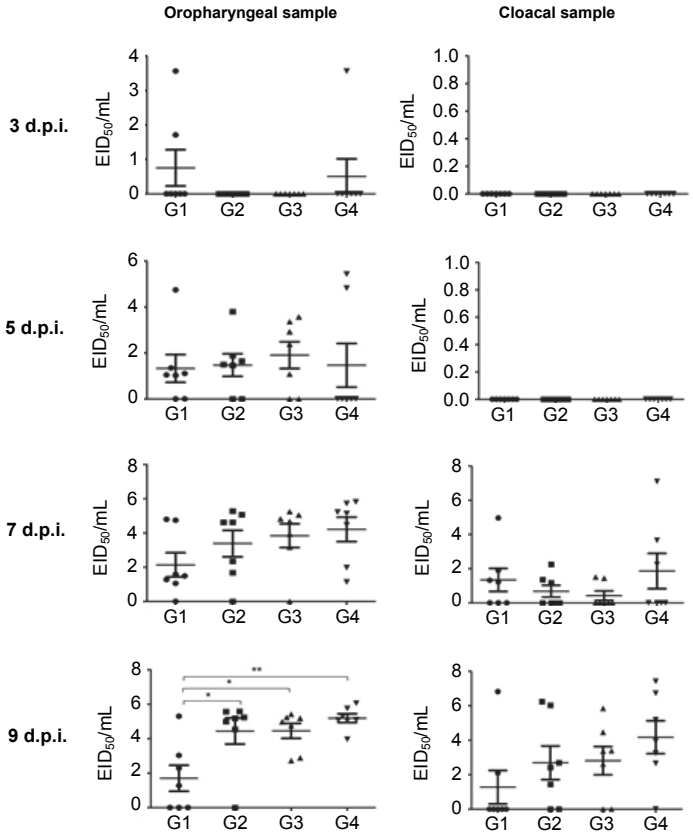

Fig. 3. Inhibition of the AIV shedding levels after the oral administration of *L. paracasei* expressing the 3D8 in chicken oropharyngeal and cloacal swabs of the contact exposed (indirectly challenged) group. Three-week-old specific-pathogen-free (SPF) chickens were housed in each containment cage in a biosafety level 2 (BSL2) animal facility. Seven chickens were intranasally challenged with 100 µL of $10^{7.5}$ EID_{50}/mL H9N2 virus. Oropharyngeal and cloacal swab samples were harvested at 3, 5, 7 and 9 days post-inoculation (dpi) from the contact exposed chickens and the viral RNA was quantified by real-time RT-PCR. Mean viral shedding titres were calculated. Data bars represent the mean ± standard error

Rohani et al. (2009) reported that virus excretion mainly occurs through the oropharyngeal and cloacal routes, resulting in transmission not only by direct contact with infected hosts but also by indirect contact with infected hosts as well as indirectly through contaminated feeding grounds and surface waters. According to Byun et al. (2017), 3D8 scFv transgenic chickens exhibited reduced viral shedding levels in the contact transmission group in oropharyngeal and cloacal swab samples after challenge with avian influenza virus. In addition, Lee et al. (2019) reported that infectious bronchitis virus levels were significantly decreased in the 3D8 scFv transgenic chickens as compared to those in the contact transmission group. These findings were in agreement with our present research, in which *L. paracasei*-secreting 3D8 administration contact transmission chicken groups exhibited a significant reduction in viral shedding when compared with other groups.

The directly infected group showed similar viral RNA transcription level among the four treatment groups such as *L. paracasei* secreted 3D8 scFv, *L. paracasei* anchored 3D8 scFv, *L. paracasei*, and the control group (Fig. 2). However, the group fed 3D8 scFv-secreting *L. paracasei* had significantly reduced oropharyngeal virus shedding compared with the other three groups at 9 dpi (Fig. 3). In the present study, the group administered 3D8 scFv-secreting *L. paracasei* showed reduced viral shedding levels as compared to the contact transmission group. Previously Hoang et al. (2015) reported that codon optimisation also improved the expression of 3D8 scFv *L. paracasei* – secreting to reduce murine norovirus infection (MNV). Our findings also have important implications for characterising the antiviral properties of 3D8 scFv-secreting *L. paracasei* in chickens. ScFvs such as 3D8 scFv that are formed by a single polypeptide are advantageous because of their small size, resistance to acid and heat, and ease of expression with an intact spatial structure. These properties make these scFvs suitable for the treatment of infections (Hoang et al., 2015). Although the mechanism of action of probiotics is not fully understood, the protective effect of the antibody-expressing *L. paracasei* in our system confirmed its antiviral activity. The advantages of using genetically modified *L. paracasei* include cost-efficient production, a long shelf life when lyophilised, simple logistics for distribution, and ease of administration (Pant et al., 2006; Hoang et al., 2015). In conclusion, the results obtained in the present study demonstrate that avian influenza virus transmission was suppressed in chickens fed *L. paracasei* expressing the 3D8 scFv protein. Therefore, the 3D8 scFv protein enhances probiotic activity against influenza virus transmission and is an effective antiviral protein that should be considered as an alternative treatment in the poultry industry. However, only few reports are available on the antiviral effect of the 3D8 scFv protein in chickens and, thus, further scientific investigations are needed.

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