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In vitro screening of immunomodulatory properties of synbiotics in chicken DT40 cell line*

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Synbiotics (prebiotic & probiotic) are used to stimulate the proper composition of the intestinal microflora in farm animals. One of the desired properties of synbiotics is connected with the immunostimulatory potential that allows for maturation of the immune system and regulation of its functions. This study aimed at selecting synbiotics with the most pronounced immunostimulatory properties, expressed by regulation of immune-related genes. For this purpose we used an *in vitro* assay based on the DT40 cell line stimulation with different synbiotics and downstream analysis of gene expression. We used a combination of three prebiotics: RFO, inulin, Bi²tos, and three probiotic bacteria strains of *Lactococcus lactis.* Stimulation was carried out at 37°C and in a 5% CO_2 atmosphere for 9 hours. Downstream analysis of gene expression was performed at the mRNA level, using the RT-qPCR method. Each of the 20 analysed genes belonged to (1) cytokines/chemokines/ regulatory molecules regulated by pre- and probiotics (i.e. IFN-γ, IFN-β, IL-4, IL-6, IL-8, IL-12p40, STAT4, CD3, CD80; iNOS), (2) the TLR2 signalling pathway (TLR2, MyD88) or (3) genes associated with the response of chickens to molecular patterns derived from gram-positive bacteria, i.e. lipoteichoic acid (MAPK8IP3, MAP2K4, MAP2K3, ITGB4, KLHL6, UNC13D and CARD11). The combination of the prebiotic inulin and probiotic *Lactococcus lactis* subsp. *lactis* SL2 provided the strongest regulation of the immune-related genes, which proves the immunostimulatory potential of this synbiotic.

KEYWORDS: chicken / gene expression / *in vitro* model /prebiotic / probiotic

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Intestinal microflora plays an essential role in the modulation of chicken immune responses [Yegani *et al*. 2008]. For example, it protects the intestine from infections, including different types of *Salmonella*, and it also has a positive effect on the chicken growth rate [Nurmi and Rantala 1973, Goren *et al*. 1988]. The composition of the gut microflora is shaped in the last days of embryogenesis. Post hatching, the chick leaves the closed and sterile egg shell structure and becomes exposed to pathogenic microorganisms, such as *Clostridium* and *Salmonella* [Dankowiakowska *et al*. 2013]. In order to provide an early protection from the food-borne pathogens, the intestinal microflora of chicks can be modulated *in ovo* with prebiotics and/or synbiotics. In this way the microbiome composition may be enriched with beneficial bacteria strains that provide protection against pathogens through the mechanism of competitive exclusion [Edens *et al*. 1997, Villaluenga *et al*. 2004].

The *in ovo* method of delivery is based on injection of a bioactive substance into the chicken embryo at different stages of embryonic development [Ebrahimnezhad *et al*. 2011, Pilarski *et al*. 2005, Cheled-Shoval *et al*. 2011]. So far it has been successfully used to deliver prebiotics [Bednarczyk *et al*. 2011] and synbiotics [Slawinska *et. al*. 2014a, Slawinska *et al*. 2014b] into 12-day-old embryos. Prebiotics are non-digestible food ingredients that can selectively stimulate growth of endogenous bacteria, such as lactobacilli and bifidobacteria, which benefits the host [Gibson and Roberfroid 1995]. Probiotics are defined as biopreparations containing living cells or metabolites of stabilized autochthonous microorganisms that optimize the colonization and composition of the gut microflora in both animals and humans. A synergistic composition of prebiotics and probiotics is defined as synbiotics.

By definition, appropriately designed synbiotics should express synergistic effects of each compound they contain [Bielecka *et al*. 2002]. However, not all of them elicit immunostimulatory properties in the host organism. Therefore, it is crucial to first perform an adequate evaluation to assess the effectiveness of the synergistic interaction between both synbiotic compounds and the host organism. The screening methods are based on *in vitro* assessment, followed by comparative *in vivo* testing performed on living animals [O'Sullivan 2000]. *In vivo* examination of pre- and probiotic properties of bioactive compounds is time-consuming, labour-intensive and requires large numbers of animals. Therefore, *in vitro* assays should be developed to allow for fast and informative screening in order to preselect the best performing synbiotics [Koenen *et al*. 2004]. One type of *in vitro* models is a microbiological one that aims to assess synergistic interactions between pre- and probiotics themselves [Saulnier *et al.*, 2008]. Another experimental *in vitro* model is based on the analysis of interactions between synbiotics and host cells using eukaryotic cell lines, in order to characterize the type of responses of host cells to stimulation. For example, this approach facilitated determination of immunostimulatory properties of *Lactobacillus acidophilus* in primary cultures of chicken spleen and cecal tonsil mononuclear cells [Brisbin *et al*. 2008], as well as the ability of 46 different strains of *Lactococcus lactis* to induce production of cytokines in the murine macrophage-like cell line [Suzuki

et al. 2008]. Moreover, the *in vitro* model helped to assess the positive role of FOS-inulin and β 1-4 mannobiose in the enhanced killing of *Salmonella enteritidis* by chicken macrophages [Ibuki *et al*. 2011, Babu *et al*. 2012].

Our proposed *in vitro* model is based on the DT40 cell line, which is a chicken lymphoid cell line derived from a B-cell lymphoma developed in the bursa of Fabricius of a female Leghorn chicken infected with avian leukosis virus (ALV) [Baba *et al.* 1985, Baba and Humphries 1984]. DT40 is a B cell-like population; therefore it accounts for the role of B cells as antigen-presenting cells and their abundance in the gastrointestinal tract. DT40 cells express the surface B-cell antigen receptor (BCR; IgM isotype) and they are used in such diverse fields as B cell antigen receptor (BCR) signalling, cell cycle regulation, gene conversion and apoptosis [Winding and Berchtold 2001]. In our case the DT40 cell line is supposed to mimic - in a very simplified way - the environment of immune cells of the intestinal tract stimulated with beneficial microbiota.

The study presented here aimed to propose the optimal composition of the synbiotics, based on their immunostimulatory properties confirmed by *in vitro* testing. Usefulness of the *in vitro* test for preselection of the best performing synbiotic is further discussed.

Material and methods

Composition of synbiotics

Three prebiotics, both in-house and commercially manufactured, were selected for the initial *in vitro* screening. They included RFOs [in-house, extracted according to Gulewicz *et al*. 2000], inulin from *Dahlia tubers* (Sigma-Aldrich, Schnelldorf, Germany) and Bi^{2}tos (Clasado, Biosciences Ltd., Jersey UK). Table 1 presents the origin, main bioactive compounds and the manufacturer/reference of the prebiotics panel. Probiotic bacteria strains were derived from the collection of the Institute of Biochemistry and Biophysics (IBB) of the Polish Academy of Sciences in Warsaw, Poland. Prebiotics and probiotics were selected in a previous experiment and described elsewhere [Bednarczyk *et al.* 2013]. Combinations of the synbiotics used in this experiment are presented in Table 2.

Table 1. Overview of prebiotics used for selection

Prebiotic	Source	Main bioactive compound	Manufacturer
RFOs	Lupine seeds	Raffinose	In-house[1]
Inulin	Dahlia tubers	Fructan	Sigma-Aldrich
Bi^{2}tos	Lactose	Galactooligosaccharides	Clasado

[1]According to Gulewicz *et al*. [2000].

Table 2. Combinations of synbiotics used for chicken lymphocyte B stimulation *in vitro*

Group	Prebiotic[2]	Probiotic[1]
C	-	-
S1	RFOs	*L. lactis* subsp. *lactis* SL1
S2	Inulin	*L. lactis* subsp. *lactis* SL1
S3	Bi^{2}tos	*L. lactis* subsp. *lactis* SL1
S4	RFOs	*L. lactis* subsp. *cremoris* SC1
S5	Inulin	*L. lactis* subsp. *cremoris* SC1
S6	Bi^{2}tos	*L. lactis* subsp. *cremoris* SC1
S7	RFOs	*L. lactis* subsp. *lactis* SL2
S8	Inulin	*L. lactis* subsp. *lactis* SL2
S9	Bi^{2}tos	*L. lactis* subsp. *lactis* SL2

C – control group, unstimulated; S1-S9 – synbiotic combinations; RFOs (raffinose family oligosaccharides), inulin, Bi^{2}tos – 5mg/well.
[1]100µl of undiluted overnight culture of *Lactococcus lactis* (~3x10^7 of bacteria), thermally deactivated.

In vitro stimulation of chicken DT40 cell line with selected synbiotics

The DT40 cell line (DSMZ, Braunschweig, Germany) was propagated using 80% Advanced RPMI 1640 medium (Invitrogen, Carlsbad, US) and 20% Foetal Bovine Serum (Biological Industries, Beit-Haemek, Israel) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 4.5 g/L glucose, 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µM mercaptoethanol at 37°C in a 5% CO_2 atmosphere. Cell culture tubes (TPP, Trasadingen, Switzerland) were used for cell propagation. The total number of 5 ×10^6 cells, with viability of at least 95%, were seeded on 6-well plates of 2 mL. At 16 hrs before the stimulation the cells were transferred into another medium (low-FBS) (Advanced RPMI 1640 supplemented with 2% FBS, 2 mM L-glutamine and 4.5 g/L glucose) in order to turn them into a passive stage, more prone for the treatment.

Prior to stimulation, the overnight bacterial cultures were prepared by inoculation of the GM17 broth with different strains of *Lactococcus lactis* and overnight incubation at 28-30°C. In order to standardize the concentration of the bacteria in the liquid culture, serial dilutions were plated on agar plates and incubated for three days at 28-30°C until the colonies were grown. The colonies were counted to calculate CFU/ml. OD was measured at the same serial dilutions of the bacteria at a 600nm wavelength. The standard curve was prepared once and for all the analyses the bacteria were standardized to the concentration of 3x10^7 based on the OD measurements. At the day of analysis fresh overnight cultures were pelleted at 6000 rpm and washed with PBS. Afterwards, bacterial pellets were resuspended with 50 mg/ml prebiotic solution (RFOs, inulin or Bi^{2}tos) and thermally deactivated at 95°C for 5 min.

Stimulation was performed by inoculation of the DT40 cells with 100 µL of the synbiotic solution. The control wells were inoculated with 100 µL low FBS medium (negative control). Each experimental and control group was represented by three biological replicates (with the well as a replicate). Stimulation was carried out at 37°C in a 5% CO_2 atmosphere for 9 hours. The incubation time was selected based on the prior series of time point experiments ranging from 3 to 24 hrs post-treatment. The expression of IL-6 and IFN-γ genes was used to select the time point with the strongest gene expression regulation (9hrs), which was used for this experiment (data not presented).

RNA extraction, RT-qPCR

Directly post-stimulation the B cells were harvested (1000xg, 5 min) and the total RNA was isolated (EURx, Gdansk, Poland). The concentration and purity of RNA samples were assessed with a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA), while rRNA band integrity was determined by 2% agarose gel electrophoresis. The RNA samples at the average concentration of ~500ng/ul; $A_{260/280}$ ratio > 1.8; $A_{260/280}$ ratio >2 and high integrity of 18S and 28S rRNA bands were subjected to two-step reverse transcription-quantitative PCR (RT-qPCR). First, cDNA was synthesized from 5 µg of the total RNA using a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific/Fermentas, Vilnius, Lithuania), following the manufacturer's recommendations. Prior to qPCR amplification, cDNA was diluted to 70 ng/µl. RT-qPCR reactions were conducted in the total volume of 20µl with 5x *HOT FIREPol* EvaGreen qPCR Mix (Solis BioDyne, Tartu, Estonia), 1 µM of each primer and 4 µl of diluted cDNA. Each RT-qPCR reaction was carried out in three individual biological replicates (with the well as a replicate) and two technical replicates. Thermal cycling and recording of real-time fluorescence emission spectra were performed in a LighCycler 480 (Roche Diagnostics, Basel, Switzerland), using the following thermal programme: 15min pre-incubation at 95°C, amplification (40 cycles): 10s denaturation at 95°C, 15s annealing of the primers (temperatures are given in Tab. 3) and 30s primer extension at 72°C (the data acquisition step). The melting curve analysis was performed immediately after the amplification protocol under the following conditions: 5s denaturation at 95°C, 1min annealing at 65°C and ramping the temperature rapidly from 98 to 40°C (with a ramp rate of 0.11°C/s and continuous data acquisition).

Table 3 shows the list of genes analysed in this study, along with RT-qPCR primers and annealing temperatures. The selection of the target genes was based on the literature [e.g. Brisbin *et al*. 2010, Sato *et al.* 2009l, Hong *et al.* 2006] and/or our prior research [e.g. Slawinska *et al.* 2014]. Each of the 20 genes selected for the analyses with RT-qPCR belonged to (1) cytokines/chemokines/regulatory molecules regulated by prebiotics and probiotics (i.e. IFN-γ, IFN-β, IL-4, IL-6, IL-8, IL-12p40, STAT4, CD3, CD80; iNOS), (2) the TLR2 signaling pathway (TLR2, MyD88) or (3) genes associated with the response of chickens to the molecular patterns derived from gram-positive bacteria, i.e. lipoteichoic acid [Siwek *et al*. 2015] (MAPK8IP3,

Table 3. Primer sequences used in RT-qPCR

Gene	Primers sequences (5'→3')	Temp[1]	Reference[2]
IFN-γ	F: ACACTGACAAGTCAAAGCCGC R: AGTCGTTCATCGGGAGCTTG	58°C	Brisbin *et al*. 2010
IFN-β	F: ACCAGATCCAGCATTACATCCA R: CGCGTGCCTTGGTTTACG	58°C	Slawinska *et al*. 2014
IL-4	F: GCTCTCAGTGCCGCTGATG R: GGAAACCTCTCCCTGGATGTC	58°C	Slawinska *et al*. 2014
MyD88	F: TCCCGGCGGTAGACAGC R: ACGACCACCATCCTCCGACACCTT	58°C	Hong *et al*. 2006
STAT4	F: ATGCTGGCAGAGAAACTTATGGGG R: CGTACCCATCAATCCAGAGAGGAA	58°C	Brisbin *et al*. 2008
CD80	F: CCCAAGGCACGCCTGTT R: CACGTCGTCTTCTGCTGAAACT	59°C	This study
IL-8	F: AAGGATGGAAGAGAGGTGTGCTT R: GCTGAGCCTTGGCCATAAGT	58°C	Slawinska *et al*. 2014
IL-6	F: AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT	58°C	Chiang *et al*. 2009
IL-18	F: GAAACGTCAATAGCCAGTTGC R: TCCCATGCTCTTTCTCACAACA	58°C	Brisbin *et al*. 2010
IL-12p40	F: TTGCCGAAGAGCACCAGCCG R: CGGTGTGCTCCAGGTCTTGGG	65°C	Brisbin *et al*. 2010
CD3	F: CAGGGATTGTGGTCGCAGAT R: TACTGTCCATCATTCCGCTCAC	58°C	Sato *et al*. 2009
iNOS	F: TGGGTGGAAGCCGAAATA R: GTACCAGCCGTTGAAAGGAC	58°C	Hong *et al*. 2006
TLR2	F: ACTGCCTGCAACGGTCAT R: CATCAGCTTCATTGTTGGTTTCTGT	58°C	This study
MAPK8IP3	F: TGGAACACATTGAACGATCCA R: GGACGTTCCTTCCTGCTTCTC	58°C	This study
MAP2K4	F: ATGGCGCCGGAAAGGATA R: CGTCTGAGCGGACGTCATAG	58°C	This study
MAP2K3	F: CGGCTGTGTGCCGTTTC R: TTGGAATCTTGCTTCTTGTCCAT	58°C	This study
ITGB4	F: TGCAAGGACAAGATTGGCTG R: GGGTAGTCCTGCTTGGTGTCAT	58°C	This study
KLHL6	F: GGTTGAAGCCAAATGCATCA R: GCCCCACCCACAACATAAAT	58°C	This study
UNC13D	F: GGTGAAGAGCATGGAGGAAAAT R: AGATCTCCTATCACCTCCAAAAGG	58°C	This study
CARD11	F: GAAGGCCTGGATGCCTATGA R: ATGCGCCTTTCCAGAGAGAA	58°C	This study
UB[3]	F: GGGATGCAGATCTTCGTGAAA R: CTTGCCAGCAAAGATCAACCTT	58°C	Boever *et al*. 2008

[1] Primer annealing temperature. [2] In-house design primer sequences based on the transcript sequence, cross exon-exon boundaries. [3] UB – reference gene.

MAP2K4, MAP2K3, ITGB4, KLHL6, UNC13D and CARD11). The reference gene (UB, ubiquitin C) was selected based on the published comparative study on the chicken reference genes [De Boever *et al.* 2008], confirmed by our validation study using the RefFinder software for reference gene analysis (http://www.leonxie.com/referencegene) (data not presented).

Prior to data processing, melting curves were first inspected for the presence of primer-dimers or unspecific PCR products. The primers had been designed to anneal to the cDNA template at the temperature of 58°C, which was represented by a single peak at the melting curve. However, when the additional peak occurred on the melting curve, the qPCR parameters were optimized, including an increase in the annealing temperature (e.g. IL-12p40 was amplified applying the temperature of 65°C during the annealing step).

Statistical analyses

The relative quantification analysis of RT-qPCR data was performed using the ddCt method [Livak and Schmittgen, 2001]. One-way analysis of variance to detect the significance of differences between means was performed using the JMP Pro 10.0.2. software (SAS Institute, Cary, NC, USA). An unpaired Student's t-test (one-tailed) was used to determine the significance of the expression data.

Results and discussion

All genes analysed were expressed in chicken B cells, except for IL-8 (Ct>35). No template controls (NTCs) were negative. Based on the results of one-way ANOVA (Tab. 4), for 9 out of the 20 genes the means were significantly different (P<0.05). However, a post hoc test provided pairwise differences between the control and treated samples in 13 genes, presented in Figure 1. In most cases, stimulation of the DT40 cells with synbiotics resulted in an up-regulation of gene expression. Only IFNγ was significantly down-regulated by the S6 treatment (P<0.05). The genes TLR2 and IL-4 underwent most consistent up--regulation in a majority of the synbiotic-treated groups. Many of the regulated genes (i.e. IFN-β, IL-4, MyD88, CD80, IL-6, TLR2, MAP8IP3, MAP2K3, UNC13D and CARD11) were induced in the S8 treatment group in comparison to the control. The stimuli of S8 consisted of the inulin prebiotic combined with LAB strain *Lactococcus lactis* subsp. *lactis* SL2.

Table 4. Probability of effects of DT40 cell line treatment with synbiotics

Gene	*P*-value
IFNγ	0.0388
IFNβ	0.0084
IL-4	0.0039
MyD88	ns
STAT4	ns
CD80	0.0020
IL-8	ns
IL-6	0.0093
IL-18	0.0307
IL-12	ns
CD3	0.0080
iNOS	ns
TLR-2	0.0018
MAPK8IP3	ns
MAP2K4	ns
MAP2K3	0.0106
ITGB4	ns
KLHL6	ns
UNC13D	ns
CARD11	ns

Results presented as prob > F Ratio.

An *in vitro* model has been successfully used in testing of immunostimulatory properties of probiotics in humans

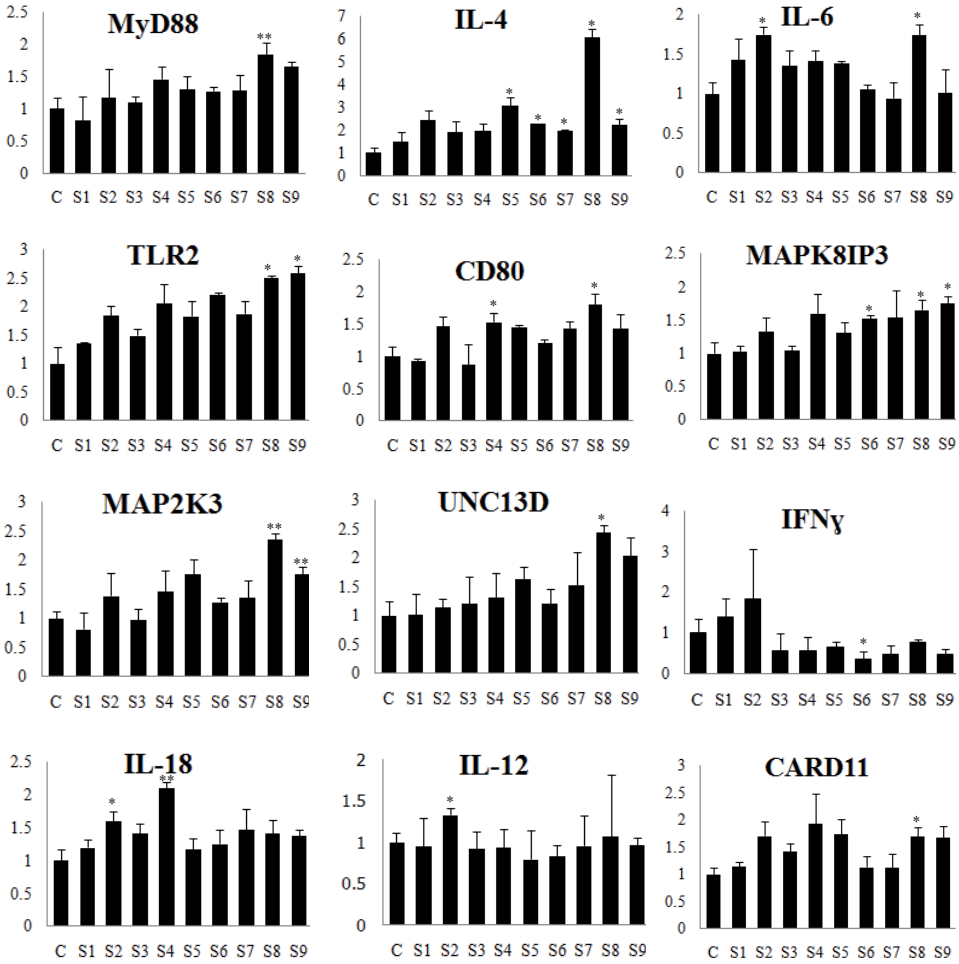

Fig. 1. Relative expression of immune-related genes in chicken B lymphocytes stimulated with different synbiotics *in vitro*. Results expressed as mean fold change over control treatment. *P<0.05; **P<0.01; statistics were conducted using a two-tailed unpaired t-test to detect significant differences between the control and synbiotic-treated pairs. Error bars represent standard error of the mean (SEM). Experimental groups are defined in Table 2.

[Pozo Rubio *et al*. 2011]. Pozo Rubio *et al.* [2011] analysed the effect of several *Bifidobacterium* strains on cytokine balance (i.e. IL-10, IL-8, IFN-γ, IFN-α) in a simulated intestinal environment. Their research led to a conclusion that the gene expression level is highly dependent on the proportions of various *Bifidobacteria*. This statement is in agreement with the results of our study, in which different strains of *Lactococcus lactis* (subsp. *lactis* SL1 or SL2, or subsp. *cremoris* SC1) were tested and gene expression levels of the host cells differed depending on the prebiotic and

probiotic combination. Bove *et al.* [2012] set up an *in vitro* model to evaluate probiotic properties of wild type and mutant strains of *Lactobacillus plantarum*. Those authors proved that the induction of immune-related genes resulted in much greater effects upon incubation with the heat-inactivated bacteria than with the live ones, which is in agreement with the *in vitro* study presented in this manuscript. Preliminary studies on DT40 cell line stimulation with live bacteria failed, due to an imbalanced growth of chicken lymphocytes and bacteria (data not presented). However, it has been proved that both live and dead cells of probiotic products can generate beneficial biological responses. Live probiotic cells influence both the gastrointestinal microflora and the immune response, whilst the components of dead cells exert an anti-inflammatory response in the gastrointestinal tract [Adams 2010].

Fructoligosaccharides, such as inulin, elicit immunomodulatory effects and therefore are used in-feed as a chicken prebiotic [Alzueta *et al*., 2010] or as a bioactive component in *in vitro* studies [Slawinska *et al*. 2014a, 2014b]. Babu *et al*. [2012] tested the influence of inulin on the ability of the chicken macrophage HD11 cell line to phagocytose and kill *Salmonella Enteritidis*, and express selected inflammatory cytokines and chemokines in an *in vitro* model. Obtained results suggest that FOS-inulin has the ability to modulate the innate immune system, as shown by the enhanced killing of *Salmonella Enteritidis* and decreased inflammasome activation (IL-1β expression was significantly lower in macrophages treated with inulin). Voght *et al*. [2013] investigated immunomodulatory effects of inulin type fructans in an *in vitro* study with human peripheral blood mononuclear cells (PBMCs). The study of Voght *et al*. [2013] showed also that the immune response signalling pathway is highly dependent on Toll-like receptors (TLRs) and their adapter, myeloid differentiation primary response protein 88 (MyD88). The MyD88 gene encodes a cytosolic adapter protein that plays a central role in the innate and adaptive immune response. Besides, MyD88 mediated signalling in intestinal epithelial cells is crucial for the maintenance of the gut homeostasis and it controls the expression of the antimicrobial lectin REG3G in the small intestine (GeneCards). This latter feature might be of particular interest when an inulin type fructan is used as a prebiotic. Apart from MyD88, several other genes were used in the presented study to investigate immunomodulatory effects of selected synbiotics. These genes represent a broad spectrum of immune related molecules. These include six cytokines (IL-4, IL-6, IL-12, IL-18, IFNβ and IFNɣ), one chemokine (IL-8), two membrane receptors (CD80, TLR2), two protein kinases (MAP2K3, MAPK8IP3), membrane-associated guanylate kinase (CARD11) and UNC13D.

It has been shown that lactic acid bacteria (LAB) confer health benefits as probiotics in a strain-dependent way [Kosaka *et al*. 2012]. Dong *et al*. [2012] analysed the immunomodulatory effect of several species of lactobacilli and bifidobacteria in a human peripheral blood mononuclear cell (PBMC) *in vitro* model. The cytokines that showed strain-specific modulation included IL-10, IFN-γ, TNF-α, IL-12p70 and IL-6. The *Lactobacillus* strains tended to promote T helper 1 cytokines, whereas *Bifidobacteria* strains tended to produce a more anti-inflammatory cytokine profile.

Results from the *in vitro* model should be validated *in vivo*, using an animal model. It has been shown that prebiotics and probiotics, properly matched by *in vitro* tests, confirmed their synergistic properties in *in vivo* conditions [Bielecka *et al*. 2002]. In our studies, the validation of selected bioactive substances was performed on a live chicken model with the use of the *in ovo* technology [Bednarczyk *et al*. 2011]. Synbiotics injected *in ovo* have functional immunomodulatory effects [Płowiec *et al.* 2015 , Slawinska *et al*. 2014a, 2014b]. Our previous experiment using synbiotics *in vivo* proved a significant effect of bioactive substances injected *in ovo* on gene expression in the spleen [Slawinska *et al*., 2014b]. We observed a significant up-regulation of the expression of the IL-4, IL-6, IFN-β and IL-18 genes and a down-regulation of IL-12 in the spleen of the S2 (*Lactococcus lactis* subsp. *cremoris* IBB SC1 with RFO) group of chickens (in comparison to the control). Similar gene expression patterns were detected in this experiment. IL-4, IL-6, IFN-β and IL-18 were up-regulated in the S4 group when compared to the control (C). The same synbiotic (*Lactococcus lactis* subsp. *cremoris* IBB SC1 with RFO) injected *in ovo* during embryo development influenced both the structure and development of the immune organs [Slawinska *et al*., 2014a]. The spleen index was significantly higher in chickens treated with S2 (*Lactococcus lactis* subsp. *cremoris* IBB SC1 with RFO) ($P<0.05$).

Synbiotics selected in this experiment were used in our *in vivo* study (Bogucka, personal communication) using the *in ovo* technology. A histological analysis performed on intestinal samples of the duodenum and jejunum of one-day-old chickens showed a positive effect of synbiotic S8 (*Lactococcus lactis* subsp. *lactis* IBB SL2 + inulin) on the number of goblet cells (Bogucka, personal communication). The high number of goblet cells in chickens immediately after hatching suggests good condition of the digestive track in chickens. The same histological analysis proved a beneficial influence of both synbiotics (S6, S8) on the area of intestinal villi in the newly hatched chickens (Bogucka, personal communication).

To sum up, it is crucial to ensure a proper composition of prebiotics and probiotics for further supplementation of the developing organism. One of the screening *in vitro* tests, which proved to be useful in the selection of the best performing synbiotic, is gene expression profiling, that facilitates monitoring of the immunomodulatory effects conferred by synbiotics on the host cells. This allowed for a selection of the synbiotic composition that expressed the highest level of the immune-related gene regulation in the chicken DT40 cell line, comprised of B lymphocytes. As a result, we pinpointed the S8 synbiotic (*L. lactis* subsp. *lactis* IBB SL2 + inulin) as the best performing one and recommended for *in vivo* studies in chickens, carried out with the use of *in ovo* technology [Płowiec *et al.* 2015]. Therefore, this study supported research on an animal model, in which the synergistic combination of prebiotics and probiotics has been used in the modulation of intestinal microflora during chicken embryo development.

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