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Assessing the Interplay Between Yeast Cell Wall Supplementation and Oral Salmonella Vaccines in Poultry Immunity

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Abstract

Due to the increasing concerns over antibiotic-resistant bacterial strains, implementing alternative strategies has become crucial for poultry health. The use of Mannan-oligosaccharides (MOS) from Yeast cell wall (YCW), and the administration of oral vaccines, are strategies that can be leveraged to help reduce the incidence of *Salmonella*. When MOS enhances gut innate immunity, live attenuated *Salmonella* vaccines promote the development of a specific humoral response. However, studies have demonstrated the binding between MOS and Salmonella pathogenic strains, thereby raising questions about the effect of a YCW-supplemented diet on the live attenuated strains and the efficiency of these vaccines. Here, we show that despite this phenomenon, *Salmonella* vaccination is at least as effective among chickens fed with YCW as in the control group. No major differences were found in the circulating antibody titer, nor in the cytokines' activation potential between the two groups. Furthermore, antigen-presenting cells' class II major histocompatibility was overexpressed by 59.9%, and an increased frequency of 70.6% in mature B-cells among the treated animals was detected. Our results demonstrate that a YCW-supplemented diet has a time-specific but positive effect on the animal's effective vaccine-induced protection. Therefore, even though binding between MOS and the vaccine may occur, it does not negatively impact the chicken's immune protection.

Introduction

Poultry farming represents the largest meat production sector worldwide, with over 130 million tons produced in 2023[1], accounting for approximately 40% of global meat production (OECD-FAO, 2023; FAO, 2024). Historically, antibiotics were commonly used at subtherapeutic doses as growth promoters in poultry production. However, this practice has now been formally prohibited since 2006 by legislation due to concerns over the development of antibiotic-resistant pathogens[2]. While the therapeutic use of antibiotics is still authorized when birds fall ill, there is an increasing emphasis on limiting such curative treatments. Concomitantly, the trend towards decreasing or even eliminating antibiotic use has increasingly challenged poultry health, as producers must find alternative preventive strategies. To address this concern and improve overall poultry health and productivity without relying on curative antibiotics, alternatives such as vaccines or dietary supplements are becoming increasingly prevalent in the industry[3]. This shift towards preventive measures not only benefits animal welfare but also aligns with the One Health concept. This approach aims to mitigate the emergence of antibiotic-resistant strains while improving poultry health. In this context, one of the most prominent pathogens affecting poultry is *Salmonella*. The European Food Safety Authority reported 77,486 cases of *Salmonella*-induced foodborne illness across Europe in 2023. Given *Salmonella*'s susceptibility to developing antibiotic resistance, this pathogen poses a significant health concern that must be addressed through alternative preventive strategies[4].

In both avian and mammalian species, the innate immune response serves as the primary line of defense against invading pathogens. This response is characterized by its rapidity, reactivity, and nonspecificity, involving diverse cellular components[5]. Importantly, this fundamental part of the immune system can be modulated through the administration of dietary supplements, including pro-, pre-, and postbiotics. Additionally, recent studies have revealed the potential to train cells engaged in innate responses, such as NK cells, monocytes, or macrophages, to exhibit an augmented yet non-specific immune response upon secondary pathogen exposure[6]. This training can be induced by endogenous alarmins or microbial ligands such as β-Glucans. Upon stimulation by these β-glucans, myeloid cell populations can display an enhanced, yet non-specific immune response following subsequent pathogen challenge[6,7].

Notably, postbiotic compounds play a key role in enhancing the innate immune response through their high levels of immunomodulatory polysaccharides. Specifically, Yeast Cell Wall (YCW)- derived postbiotics are rich in mannan-oligosaccharides (MOS), a glucomannoprotein known for its immunostimulatory properties. These MOS can bind to specific pili on gram-negative bacteria, such as *Salmonella*, which would otherwise attach to the gut wall during colonization[8]. Previous investigations have demonstrated the positive impact of mannan-supplemented diets on chicken gut health, enhancing this binding and leading to significant improvements in host well-being[9]. Among these health benefits, MOS can improve the composition of the gut microbiome and the chicken's physical characteristics[10]. More importantly, mannan can also bind to C-type lectins, a specific superfamily of glycan-binding receptors on Antigen Presenting Cells (APCs), such as dendritic cells and macrophages. This family of receptors is linked to the recognition of a variety of pathogenic motifs and priming of the innate immune response. They are known to be activated when bound to MOS, enhancing this antigen phagocytosis as well as the overall activity of these APC, thus potentially augmenting the chicken's immunity[11].

Postbiotics compounds, such as those derived from YCW, stimulate gut leukocytes and the innate immune response, whereas vaccines primarily involve the humoral immunity, inducing the production of pathogen-specific antibodies. Most poultry vaccines are administered either orally or subcutaneously and aim to trigger an adaptive immune response by activating and differentiating B-cells

into antibody-secreting plasma cells. This study focused on an oral vaccine using live attenuated *Salmonella* strains. Upon reaching the ileum, this vaccine will either be detected within the lumen by dendritic cells or cross the intestinal epithelium through M cells to access the *lamina propria*[12]. The live attenuated bacteria will then be primarily phagocytosed by macrophages before being processed for antigen presentation by APCs. After migrating to germinal centers, such as the Peyer's patches or the tonsils, these APCs will activate naïve T-cells by presenting the antigen through the class II major histocompatibility complex (MHC II). Naïve T-cells will subsequently differentiate into helper T-cell subsets, including Th1 and Th17 lineages. Th17 T-cells are known to activate CD8 + cytotoxic T-cells, leading to the killing of infected cells via the secretion of granzymes and perforins. Meanwhile, Th1 lymphocytes, in association with APCs, will stimulate B-cell proliferation and differentiation into antibody-producing plasma cells, thereby generating a humoral response specifically directed against *Salmonella*[13].

It has been shown that the components of the yeast cell wall have adhesive properties to bacteria, primarily through type 1 fimbriae-dependent adhesion, where bacterial fimbriae containing the FimH adhesin interact with mannose structures present in the yeast cell wall, though additional factors beyond just mannan content may contribute to this binding mechanism[9,14]. This adhesion of pathogenic bacteria to YCW serves as a competitive exclusion strategy, effectively preventing these microorganisms from attaching to intestinal epithelial cells and thereby reducing their colonization potential and pathogenicity in the host (Spring et al., 2020; 14). Although both YCW and vaccination are useful in reducing *Salmonella* prevalence, one question remains. This bacterial adhesion to YCW improves the elimination of pathogenic bacteria in the intestinal lumen; nevertheless, this adhesion may also occur between the MOS contained in the YCW and an oral vaccine in which the antigen is a live attenuated bacterium. Therefore, the potential interaction between the YCW and the vaccine might impact the vaccine's efficiency. This study aims to investigate this interaction and the effect of a *Salmonella* oral vaccine on the immunity of an animal fed with a YCW-supplemented diet.

Results

Adhesion between YCW and pathogenic Salmonella strains

A preliminary study was conducted to quantify the adhesion of the YCW product to a pathogenic strain of *Salmonella enterica typhimurium* isolated from a turkey farming environment. Using a microplate-based test assay, the quantitative adhesion between bacteria and yeast cell wall was determined by measuring the concentration of adhering bacteria via ATP-bioluminescence. With this method, the binding capacity was determined to be 3.10^4 CFU/µg of YCW (Fig. 1).

Leukocyte profiling across tissues post-vaccination in laying hens

This study presents a detailed immunophenotyping of PBMC and LPL compartments in laying hens following multiple standard vaccinations. In the gating strategy presented in **Fig. 2A**, viable leukocytes were identified as single Zombi$^+$/CD45$^+$ events and subsequently gated based on their CD3 and Bu1 expression. Three distinct leukocyte populations were identified: T-cells as Bu1$^-$/CD3$^+$, B-cells as Bu1$^+$/CD3$^-$, and myeloid cells as Bu1$^-$/CD3$^-$. Within these populations, T-cells lacked MHC II expression, B-cells demonstrated MHC II positivity, and various subpopulations of myeloid cells were further delineated based on MHC II expression. Notably, all MHC II$^+$ myeloid cells were also FSC-A^{high} and therefore classified as either monocytes in blood samples or macrophages in tissue samples from the *lamina propria* or the tonsils[15,16]. Additionally, two other subpopulations were identified among myeloid cells, both MHC II$^-$, but either FSC-A^{high} or FSC-A^{low}. We hypothesized that the MHC II$^-$/FSC-A^{low} subset corresponds to thrombocytes, given their small and nucleated features in avian species, while the MHC II$^-$/FSC-A^{high} subpopulation may represent innate lymphoid cells (ILCs)[17]. Regarding the T-cells, these findings revealed a well-defined compartment using only three markers: TCRγδ, CD8β, and CD4. This facilitated the identification of both CD8αα and CD8αβ cytotoxic T-cells, helper T-cells, and two distinct TCRγδ subpopulations (Fig. 2A).

Analysis of leukocyte distribution across the three main compartments revealed notable organ-specific differences. In the *lamina propria*, myeloid cells are the predominant compartment, comprising 45% of leukocytes, followed by T-cells at 33% and B-cells at 22%. In contrast, the tonsil exhibited fewer than 5% myeloid cells, resulting in an overrepresentation of T-cells and B-cells, at 52% and 44%, respectively. Lastly, peripheral blood mononuclear cells (PBMCs) exhibited a well-balanced ratio between the T-cell and B-cell compartments, constituting 15% and 18% of the total leukocytes, respectively. Notably, this was accompanied

Figure 2: Immunophenotyping of 12-week-old vaccinated chickens. A. *Gating strategy used to identify the different leukocyte populations.* **B.** *Leukocytes distribution between the B, T, and myeloid lineages in the lamina propria, the blood, or the tonsils.* **C.** *Leukocytes distribution inside the myeloid compartment in the lamina propria, the blood, or the tonsils.* **D.** *Leukocytes distribution between the T-cells'*

subpopulations in the lamina propria, the blood, or the tonsils by a strong predominance of the myeloid compartment, which accounted for 66% of the total leukocytes **(**Fig. 1B**).**

The composition of the myeloid compartment displayed marked variations across organs. Within the *lamina propria*, APCs accounted for 69% of myeloid cells, establishing themselves as the most abundant immune subpopulation of the organ. It is to be noted that ILCs, although not strictly classified within the myeloid lineage, were included in this analysis for clarity purposes, as an antibody specifically targeting NK cells has not yet been developed in poultry[17]. The tonsil's myeloid compartment was not investigated further due to its minimal representation, accounting for only 4% of the organ. Interestingly, thrombocytes were the most frequent cell type among PBMC, corresponding to nearly 90% of the blood's myeloid compartment **(Fig. 2C**).

Finally, immunophenotyping facilitated an in-depth characterization of the T-cell compartment (Fig. 2D). Nearly all known T-cell subpopulations were identified, aligning closely with existing avian literature[17]. However, limitations in the antibody panel were encountered, specifically the absence of TCRαβ and CD8αα antibodies, necessitating the identification of certain subpopulations through negative selection.

YCW improves the innate response to the vaccine

Upregulation of the MHC II expression

Myeloid populations play a key role in the innate immune response and, therefore, are essential for pathogen recognition and the initiation of downstream immune processes. To explore whether dietary supplementation with YCW influences this compartment, the frequency of myeloid cells was first assessed. Immunophenotyping demonstrated that the YCW-supplemented diet did not significantly alter the prevalence of these populations. No notable differences were detected between treated and control animals across the *lamina propria*, tonsils, and blood (Fig. 3A). Unfortunately, heterophils could not be identified, and APCs could not be further characterized. Previous analysis revealed a lack of specificity of the monocytes-macrophages marker (clone KUL01). Therefore, macrophages, monocytes, and dendritic cells were classified as APCs, FSC-A^{high}/MHC II^{+}, even though this population is

Figure 3: Characterization of the myeloid compartment among YCW-fed animals. A. *Myeloid cells frequency, gated for viable single leukocytes CD45^{+}/Bu1^{-}/CD3^{-}.* **B.** *Evolution of the MHC II MFI calculated by geometric mean over time in the blood APCs.* **C.** *MHC II MFI of blood APCs depending on the treatment. D. FlowJo image of the APCs high and low groups.* **E.** *Distribution of the Lamina propria APCs based on their MHC II expression. Data are presented as means +/- SEM (N = 5–10). Mann-Whitney test (A, C) or One-way ANOVA (B, E) was applied for statistical analysis (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).*

supposed to be mainly constituted of monocytes or macrophages, depending on the tissue. Additionally, a YCW-supplemented diet did not alter the relative levels of thrombocytes; however, due to the absence of a thrombocyte-specific marker, this subpopulation may have been mixed with residual cellular debris, precluding further investigation (see **Supplementary Figure S1**).

However, some changes in MHC II expression were observed in APCs. This antigen presentation complex is crucial for the activation of T-cells, and therefore to the induction of the adaptive immune response[18]. Blood monocytes exhibited a significant 86% decrease ($p < 0.0001$) in their MHC II expression at both weeks 12 and 24 compared to week 8 (Fig. 3B). This reduction in MHC II levels was observed in both the treated and control animals, without any treatment-related effect (Fig. 3C). In contrast, APCs from the *lamina propria* demonstrated treatment-specific changes in their MHC II expression. Subpopulations of APCs were defined by differences in MHC II levels: MHC II^{high} and MHC II^{low} (Fig. 3D). At week 12, animals receiving the YCW-supplemented diet exhibited a significantly increased proportion of APCs with MHC II^{high} expression, reaching 25.1%, compared to 15.7% in the control group ($p = 0.0283$) (Fig. 3E). These results indicate that the YCW-supplemented diet increases APC activation, enhancing their MHC II expression.

Priming of the innate response

To evaluate the functional effects of the YCW-supplemented diet on the immune response, the mRNA expression levels of several key cytokines were quantified in PBMCs after a 6-hour *in vitro* stimulation with *S.* Typhimurium antigens. The selection of cytokines included molecules involved in diverse immune functions: pro-inflammatory mediators such as IL-8, IL-1β, and IFN-γ; immunoregulatory and anti-inflammatory cytokines, including IL-10; and a marker of cytotoxic activity, inducible nitric oxide synthase (Nos2).

Following stimulation with *S.* Typhimurium antigens, a robust cytokine response was observed in PBMCs from all animals and across all investigated conditions. Notably, the comparison between the control and YCW-treated groups revealed no significant differences in cytokine mRNA production for any of the tested markers, including IL-8, IL-1β, IFN-γ, IL-10, and Nos2. Both groups demonstrated comparable activation patterns, with the stimulation eliciting a substantial upregulation of cytokine expression across these diverse immune mediator classes (Fig. 4).

These findings suggest that while the *S.* Typhimurium antigen successfully triggered an innate immune response in PBMCs, the YCW-supplemented diet had no detectable effect on the induction of this response. These results imply that the YCW supplementation did not influence the activation potential of the innate immune system in laying hens following antigenic challenge, maintaining a similar immunological capacity between treated and control groups.

Figure 4: Cytokine production upon in vitro stimulation. *Leukocytes were stimulated with S. Typhimurium antigen for 6 hours, and cytokines' mRNA were quantified by Taqman qPCR in both the lamina propria* **(A)** *and the blood* **(B)** *at 24 weeks for control (gray) and YCW-treated (orange) animals. Data are presented as means +/- SEM (N = 6). 2-way ANOVA was applied, followed by post hoc test for statistical analysis (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).*

Modulation of T-Cell dynamics and subtypes by YCW supplementation

Minor YCW-induced changes in T-Cell subtypes

Building on the analysis of the innate immune response, the impact of the YCW-supplemented diet on the adaptive immune system was then evaluated, with a specific focus on T-cell frequencies and subpopulation dynamics. T-cells were segregated based on their T-cell receptor (TCR), either TCRαβ or TCRγδ. Among TCRαβ T-cells, cytotoxic T-cells (CD8αα and CD8αβ), as well as helper T-cells ($CD4^+/CD8^-$), were identified (Fig. 5A).

Traditionally, T-cells are characterized by their TCR type and associated coreceptors. TCRγδ T-cells exhibit a more innate-like immune profile and are known for their migratory behavior during bacterial infections[19]. Despite these properties, their functional response aligns more closely with T-cell-mediated mechanisms. Upon recognizing cellular stress or infection-induced modifications, TCRγδ cells secrete effector molecules like granzymes and perforins to eliminate compromised cells[12]. In contrast, TCRαβ T-cells are considered part of the adaptive

Figure 5: Characterization of the T cell subpopulation. A. *Classification of T cells based on their TCR and coreceptor (left) and representative FlowJo images (right).* **B.** *T cell frequency, cells gated for viable single leukocytes $CD45^+/Bu1^-/CD3^+$.* **C.** *Distribution of the T cells into αβ or γδ TCR.* **D.** *Coreceptors frequency among TCR $γδ^+$ T cells.* **E.** *Coreceptors frequency among TCR$αβ^+$ T cells. Data are presented as means +/- SEM (N = 5–10). Mann-Whitney test was applied for statistical analysis (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).*

immune system, requiring antigen presentation for activation and proliferation. TCRαβ populations can be divided into cytotoxic (CD8αα or CD8αβ) and helper ($CD4^+$) subsets. Cytotoxic T-cells eliminate infected cells through mechanisms similar to TCRγδ cells, while helper T-cells primarily act to stimulate other immune cells, including macrophages, cytotoxic T-cells, and B-cells, driving their differentiation into plasma cells that produce antibodies[20]. Despite their functional importance, the distinctions between CD8αα and CD8αβ subsets remain poorly understood and minimally characterized in the existing avian literature.

Immunophenotyping demonstrated no significant differences in overall T-cell frequencies between control and treated animals across the three tested organs, except for one notable finding in the tonsil at 12 weeks (Fig. 5B). At this time point and in this organ, a YCW-supplemented diet was linked to a 44% decrease ($p = 0.0286$) in T-cell frequency. However, this reduction corresponded with a simultaneous 36 percentage-point increase in B-cell frequency in the same organ, potentially reflecting a proportional redistribution of immune cell populations.

Regarding TCRs' prevalence, no significant differences were observed for the relative frequencies of TCRαβ or TCRγδ T-cells between control and treated animals, except in PBMCs at 12 weeks. A substantial 10% increase ($p = 0.0326$) in TCRαβ T-cell frequency was observed in PBMCs, resulting in a proportional decrease in the TCRγδ population (Fig. 5C). This reduction was associated with a decrease of both TCRγδ/CD8αα and TCRγδ/CD8αβ subsets in PBMCs by 27% ($p = 0.036$) and 42% ($p = 0.0162$), respectively (Fig. 5D). Additionally, a 23% decrease ($p = 0.0454$) in TCRγδ/CD8αα T-cells was observed among the LPLs at 24 weeks.

Interestingly, TCRαβ T-cell subsets showed additional variation. At 24 weeks, helper T-cell ($CD4^+$) frequencies in the lamina propria exhibited a significant 20% decrease ($p = 0.0142$), while cytotoxic CD8αα T-cells showed a 28% increase ($p = 0.0163$) (Fig. 5E). No other differences were identified for the remaining TCRαβ T-cell subpopulations.

Overall, these findings demonstrate that while a YCW-supplemented diet does not induce widespread changes in T-cell profiles or TCR distribution, specific time- and tissue-dependent effects may reveal nuanced modulations in T-cell dynamics and functionality.

Identical activation potential of polarization pathways

To further investigate the effects of a YCW-supplemented diet on T-cells, the mRNA expression of key cytokines associated with adaptive immune responses was quantified in LPLs (Fig. 6A) and in PBMCs (Fig. 6B). Leukocytes were activated *in vitro* using a cocktail of PMA and ionomycin for 3 hours to induce T-cell-based cytokine production. The analyzed cytokines included IFN-γ, IL-17, IL-22, and IL-10, which are crucial indicators of the ongoing T-cell polarization pathways. IFN-γ serves as a hallmark cytokine of Th1 polarization, IL-17 and IL-22 are primarily associated with the Th17 polarization pathway, and IL-10 is an anti-inflammatory cytokine linked to Treg responses[21].

Following PMA/ionomycin stimulation, a strong cytokine response was induced across all conditions in both PBMCs and LPLs, demonstrating effective activation of T-cell responses *in vitro* (Fig. 6). When comparing cytokine mRNA levels between the YCW-treated and control groups, no significant differences were observed for any of the examined cytokines. Both groups exhibited comparable activation patterns, indicating consistent stimulation across the Th1, Th17, and Treg polarization pathways. These results suggest that a YCW-supplemented diet does not markedly influence the cytokine polarization profile of T-cells under the tested conditions.

YCW increases B-cell activity

Increased maturity among B-cells

Given the critical role of B-cells in humoral immunity through antibody production and antigen presentation, the next step was to assess how the YCW-supplemented diet affected their distribution, frequency, and maturation. Immunophenotyping analysis revealed notable alterations in the B-cell compartment induced by the YCW-supplemented diet. In treated animals, a reduction in B-cell frequency was observed in the *lamina propria*, with decreases of 20% and 10% detected at 8 and 12 weeks, respectively. Conversely, the YCW diet led to a substantial increase in B-cell frequency in the tonsils at 12 weeks, with a significant rise of 36 percentage points compared to control animals ($p = 0.0286$), although data for the tonsils at 8 weeks were unavailable. No significant differences in B-cell frequencies were identified at 24 weeks or in PBMCs (Fig. 7A). These observations raise the intriguing possibility that the YCW-supplemented diet may have promoted the migration of B-cells from the lamina propria to secondary lymphoid organs such as the tonsil.

Beyond these shifts in B-cell frequency, differences in B-cell maturation states were also noted. The B-cell marker Bu1, also referred to as chB6, is associated with a more immature phenotype[22]. During the immunophenotyping, two well-established B-cell subpopulations were identified, corresponding to either a more mature phenotype ($Bu1^{low}$) or a more immature one ($Bu1^{High}$) (Fig. 7B). Interestingly, in the tonsil at 12 weeks, 85% of the B-cells isolated from YCW-fed birds exhibited low Bu1 expression ($Bu1^{low}$) compared to only 55% in control animals, suggesting increased maturation within the treated group. A similar enhancement in B-cell maturation was observed in PBMCs from treated animals, evidenced by an increase in the proportion of $Bu1^{low}$ cells at 8 and 12 weeks post-dietary intervention (Fig. 7C). These findings indicate that a YCW-supplemented diet may modulate both the tissue-specific distribution and maturation status of B-cells.

Similar surface immunoglobulins and secreted antibodies

Flow cytometry was used to verify the immunoglobulin isotype expressed on the surface of B-cells. Due to technical constraints related to the limited availability of commercial antibodies, only IgA- and IgM-positive B-cells could be identified at 24 weeks in the three compartments (Fig. 7D). Across all tested organs, no significant differences in the distribution of these isotypes were observed between the control and treated groups (Fig. 7E).

Even though no class switch in B-cell surface immunoglobulins was detected, circulating IgG levels were analyzed to assess potential differences in the systemic antibody response. Using ELISA, circulating IgG antibodies specific to *Salmonella* were quantified in the

plasma. In both treated and control groups, an increase in antibody titers was observed after each vaccine booster, with no significant differences between the groups (Fig. 7F). These findings suggest that a YCW-supplemented diet does not impact the efficacy of the *Salmonella* vaccine.

Additionally, circulating antibody levels against two other avian pathogens, Newcastle Disease Virus (NDV) and Infectious Bronchitis Virus (IBV), were evaluated following subcutaneous vaccination against these diseases. Similar antibody responses were observed between the YCW-fed and control animals across most time points, except for NDV at 18 weeks and IBV at 8 weeks. At these specific time points, animals receiving the YCW-supplemented diet exhibited significantly higher antibody titers compared to controls. (Fig. 7F).

Discussion

The present study provides valuable insights into the interplay between a YCW-supplemented diet and the efficacy of an oral live attenuated *Salmonella* vaccine. Encouragingly, despite the established capacity of YCW to bind various bacterial structures—which could theoretically interfere with the vaccine strain—our results demonstrate that this dietary supplementation had no detrimental effect on either leukocyte frequencies or their functional activation potential compared to the untreated control group. Transient modulations in certain leukocyte populations were observed, but the vaccine-induced antibody titers remained largely unchanged between the YCW-supplemented and control groups.

The study provides comprehensive immunophenotyping of leukocyte populations in laying hens following routine vaccination protocols, largely consistent with the existing avian literature. The overall cellular composition of the examined organs revealed a well-balanced distribution of immune cell types within the lamina propria[23,24]. The cecal tonsils, known to be a B- and T-cell-rich compartment, exhibited the expected paucity of myeloid cells[18,19,25]. Additionally, the peripheral blood was confirmed to be predominantly composed of nucleated thrombocytes, as previously documented in avian species[26,27]. While the immunophenotyping data presented a complete characterization of the lymphocyte landscape, the study was limited in its ability to precisely delineate the myeloid cell compartment. It is important to note that these findings require further investigation due to limitations of the commercially available antibodies for flow cytometric analysis. This technical constraint is acknowledged as a potential area for improvement in future investigations. Overall, the comprehensive immune cell profiling provides a solid foundation for understanding the baseline of chicken immune status in response to routine vaccination practices.

Among the observed transient modulations, the YCW-supplemented diet was associated with an increased expression of MHC II on APCs at 12 weeks. However, those cells exhibited a similar degree of activation in response to antigen stimulation, regardless of dietary treatment. This suggests that the YCW-supplemented diet may influence the reactivity, rather than the intensity, of the humoral immune response. This hypothesis is consistent with the antibody titer data, which showed transient increases for certain pathogens at specific time points, though these effects were relatively subtle. Additionally, the variability in leukocyte populations between different organs, such as the lack of certain myeloid cells in the tonsils and the predominance of nucleated thrombocytes in the PBMC fraction, presented methodological challenges.

In contrast, the T-cell compartment was characterized in great detail and precision. The only technical limitation was the negative selection of certain cellular subtypes, but the results were consistent with the existing avian literature, such as the observation that the TCRγδ T-cell population only contained $CD4^{-}/CD8\alpha\alpha^{+}$ or $CD4^{-}/CD8\alpha\beta^{+}$ cells[17]. Among these well-described T-cell subsets, only slight changes were identified, and the activation potential in response to PMA/ionomycin stimulation was similar between the treated and untreated animals. It is worth noting that the existing avian literature lacks a comprehensive description of these T-cell subpopulations. Given the subtle nature of the observed changes, the research team concluded that these minor alterations were unlikely to be of significant relevance to the effects of the YCW-supplemented diet on the vaccine response. Overall, the T-cell compartment was meticulously characterized, but the data revealed only minimal changes, suggesting that the YCW diet had a negligible impact on this particular arm of the immune system.

The most significant data from this study were obtained from the analysis of the B-cell compartment. The immunophenotyping revealed punctual changes, such as an increased frequency of B-cells and a higher level of maturation, which can be linked to a stronger humoral response induced by the YCW-supplemented diet. This is in accordance with both the transient increased circulating antibody titer for NDV and IBV and existing literature showing a relatively similar distribution of immunoglobulin isotypes. This also suggests that the YCW-supplemented diet did not significantly alter B-cell class switching[28]. Most importantly, the circulating antibody titers against *Salmonella* remained unchanged compared to the untreated animals. These findings indicate that a YCW-supplemented diet can enhance humoral protection without negatively impacting the efficacy of oral vaccines using live, attenuated gram-negative bacteria,

such as the *Salmonella* vaccine strain. This is a crucial observation, as the YCW's ability to bind to bacterial structures has raised concerns about potential interference with the vaccine's immunogenicity.

Despite prior immunophenotyping efforts in chickens, most studies remain limited in scope and focus. While longitudinal analyses of PBMC profiles have been conducted, and occasional cross-sectional studies have evaluated leukocyte populations in the lamina propria, no previous work has employed an integrated approach to assess immune dynamics both systemically and at mucosal sites over time[15,20,29]. This combined approach is critical because mucosal immunity in the lamina propria serves as the first line of defense against pathogens at the gut barrier, whereas systemic immunity functions to mediate responses at distant sites and maintain immune surveillance. Examining both compartments simultaneously offers a complementary view of immune kinetics, particularly for assessing the effects of dietary interventions like YCW supplementation. This study thus fills a crucial gap by investigating immune cell kinetics simultaneously at both mucosal and systemic levels, offering insights into tissue-specific evolution of poultry immunity during dietary supplementation and vaccination.

In conclusion, this study demonstrates that YCW supplementation can be safely administered alongside vaccination protocols without compromising immune response development. The vaccine-induced antibody titers remained largely unchanged in the supplemented group, which was also associated with transient modulations in leukocyte populations, indicating an improved immune reactivity (Fig. 8). These findings suggest that YCW-based supplements can be leveraged to support poultry health and productivity, even in the context of reduced antibiotic use, while enhancing poultry performance[30]. However, the study's limitations warrant further investigation to fully elucidate the complex interplay between dietary interventions, innate immune modulation, and adaptive immunity in poultry. Therefore, additional analysis elucidating the underlying mechanism, especially in an infectious background, is still required to further characterize the previous effect of YCW in response to *Salmonella* colonization[31]. As the industry shifts towards preventive strategies to address antibiotic resistance, the integration of YCW supplements with vaccination programs may offer a promising approach to enhance poultry health and mitigate antimicrobial resistance, aligning with the principles of the One Health concept.

Material and Methods

Quantitative in vitro assay to evaluate the capability of YCW to bind bacteria

In this work, we quantified the adhesion of *Salmonella enterica subsp. Typhimurium* (Labocea, Ploufragan, France) to a yeast product (Safmannan, Phileo, France) using a bioluminescence-based assay in microplates. The luminescence signal was proportional to the number of adhering bacteria, allowing for the quantitative assessment of bacterial adhesion.

The method was adapted from Ganner et al.[14]. Briefly, yeast derivatives were suspended in phosphate-buffered saline (PBS). The wells of a 96-well plate were coated with 100 µL of the yeast cell wall suspension per well and incubated for 16–18 hours at 4°C. The plate was then washed three times with PBS containing 0.0125% Tween 20 (PBS-T). Subsequently, a bacterial suspension of *Salmonella Typhimurium* grown in Tryptic Soy Broth (TSB) at the exponential phase was added to the wells. Bacteria were allowed to adhere for 60 minutes at 37°C, and the plate was then washed 6 times with PBS-T to remove non-adherent bacteria. The adherent bacteria were quantified using the BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Charbonnières-les-Bains, France). This kit is a homogeneous method for determining the number of viable bacterial cells in culture based on the quantification of ATP present. The luminescent signal is proportional to the amount of ATP, which is directly proportional to the number of cells in culture. 100 µL of the BacTiter-Glo™ reagent were added to the wells, and the luminescence was recorded using a Tecan plate reader. In parallel, serial dilutions of *Salmonella* were prepared and applied to both the microplate and an agar plate to establish a linear regression between the number of counted bacteria on the agar plate (CFU/mL) and the luminescence measured. The blank consisted of the yeast cell wall product without added test bacteria, and all the assay steps were performed to determine the background bacterial number of the yeast cell wall product. The binding control consisted of test bacteria without added yeast to determine the non-specific binding sites. Finally, for the binding control, the number of bacteria that adhered to the non-specific binding sites of the well was subtracted from the calculated data to determine the final number of specifically adhering bacteria.

Ethics statement

This study was conducted in full compliance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. All experimental procedures involving animals were reviewed and approved by the Ethical Committee of Poulpharm, Belgium, under the trial code 2024/113, ensuring adherence to the highest standards of animal welfare.

Yeast Cell Wall-supplemented diet

Animals were provided ad libitum access to a basal feed formulation representative of commercial European layer diets, formulated to meet NRC (1994) nutritional requirements for laying hens. The experimental diet was supplemented with 250 g/ton of a YCW product (Safmannan®, Phileo By Lesaffre, France) throughout the experimental period. Safmannan® is a postbiotic rich in MOS (≥ 20%) and β-glucans (≥ 20%), derived from strains of *Saccharomyces cerevisiae*.

Animal study

In all experiments, female *G. gallus domesticus* (Lohmann brown layer) newly hatched chickens provided by Poulpharm, Belgium, were used. A total of 80 animals were divided into two experimental groups, with the untreated group serving as the control and the treatment group receiving the YCW-supplemented diet.

Chickens were housed in groups of 10 on pens with a floor space of approximately 1 m²/chicken. The pen floors were covered with a 5 cm layer of wood shavings and provided with natural light supplemented by artificial lighting on a 24-hour cycle. Heating lamps were used to provide additional warmth during the initial days. Feed and water were available *ad libitum* throughout the trial. Animals did not receive any antimicrobial or anti-inflammatory medication and were vaccinated according to the schedule outlined in **Supplementary Table S1**. Chickens were sacrificed at 8, 12, or 24 weeks of age by cervical dislocation, preceded by concussion or electrocution, depending on the animal's weight.

LPL isolation

Lamina propria leucocytes (LPLs) were obtained using an adapted version of the protocol described by Baillou et al.[32]. Briefly, 10cm pieces of fresh ileum were segmented in 1cm pieces and rinsed with 5mM Corning™ EDTA (Fischer Scientific #15323591) in PBS (Fischer Scientific #14190144) + 5% FBS (Fischer Scientific #17964671) for 25min at 37°C then enzymatically digested for 45min by 120µL of collagenase (Sigma #C2139) and 100µL of DNAse1 (Sigma #D5319) in RPMI 1640 (Fischer Scientific #12027599) + 5% FBS at 37°C. Afterwards, the digested supernatant was filtered, and leukocytes were isolated through Percoll® (Sigma #GE17-0891-01) density gradient separation.

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated through Ficoll-Paque PLUS density gradient separation. Leukocytes were then washed twice by centrifugation at 1500 rpm for 7 min, and the pellet was resuspended in culture medium before plating. All steps were performed using cold PBS supplemented with Ca^{2+} and Mg^{2+} (Fischer Scientific #13492609).

Tonsillar leukocytes isolation

Leukocytes were isolated from the tonsils by smashing half of the organ through a 70µm nylon mesh strainer using the piston of a 10mL syringe, and rinsed with 50mL of cold PBS. Tubes were centrifuged for 5min at 1500 rpm, and the pellet was resuspended and washed twice with PBS + 1% FBS + 5 µM of EDTA before plating.

Cell culture and in vitro stimulation

Isolated cells were counted with trypan blue, then plated at $1x10^6$ cells per well in 96-well round-bottom plates and cultured with RPMI 1640 + 10% FBS + 1% Penicillin-Streptomycin 100x (Fischer Scientific #16478946), at 37°C, 5% CO_2. Cells were stimulated for different times with either 1X PMA/Ionomycin (Invitrogen #00-4970-93) or 10^6 heat-inactivated *S. Typhimurium*.

Flow cytometry

Immediately after isolation, leukocytes were incubated for 20 min at + 4°C with a mix of antibodies diluted at 1:100 (Table 1), then fixed with paraformaldehyde. Cells were then resuspended in PBS + 1% FBS and stored overnight at + 4°C. The next day, leukocytes were analyzed by flow cytometry using a CytoFLEX LX from Beckman Coulter Life Sciences. All analyses were done on singlet Alive $CD45^+$ events.

Table 1: Antibodies used in the immunophenotyping assay

Target	Fluorochrome	Clone	Supplier	Reference
Zombi UV	~DAPI		Biolegend	#423108
CD45c	APC	LT-40	Southern Biotech	#8270-11
CD3	AF-700	CT-3	Southern Biotech	#8200-27
CD4	PE-Cy7	CT-4	Southern Biotech	#8210-17
CD8β	FITC	EP42	Southern Biotech	#8280-02
TCRγδ	Biotin	TCR-1	Southern Biotech	#8230-08
	Streptavidin BV-785		Biolegend	#405249
MHCII	PE	2G11	Southern Biotech	#8350-09
Bu1	Pacific Blue	AV20	Southern Biotech	#8395-26
IgA	FITC	A-1	Southern Biotech	#8330-02
IgM	SPRD	M-1	Southern Biotech	#8310-1

qPCR and RNA extraction

After *in vitro* stimulations, cells were lysed in 130µL of RA1 buffer + 1.3µL of TCEP per well. Lysates were then frozen, and total RNAs extracted using RNA Extraction Kit according to manufacturer instructions (Machery-Nagel, #740466.4). Afterwards, Reverse transcription was done using High-Capacity RNA to cDNA™ Kit (Applied Biosystems™, #4387406) following manufacturer instructions.

The synthesized cDNAs were diluted to 1:2 in sterile H_2O and used as qPCR templates. Real-time PCR was performed with TaqMan™ Fast Advanced Master Mix for qPCR (Applied Biosystems™, #4444964). The Light Cycler Quant Studio 5 from Applied Biosystems™ was used to measure the relative gene expressions, which were normalized using GAPDH. The quantified genes were IFNγ (assay ID number: gg03348618_m1), IL-10 (assay ID number: Gg03358689_m1), IL-17a (assay ID number: Gg03365522_m1), IL-18 (assay ID number: Gg03337831_m1), IL-1β (assay ID number: Gg03347154_g1), IL-22 (assay ID number: Gg07159410_m1), Nos2 (assay ID number: Gg03347749_m1), and GAPDH (assay ID number: Gg03346982_m1).

ELISA

Circulating antibodies against *Salmonella*, NDV, and IBV were quantified by Enzyme-Linked Immunosorbent Assay (ELISA) following manufacturer instructions (Biocheck, respectively #CK218, #CK122, and #CK119). An additional pre-diluted control was added to ensure the quality and reproducibility of our results (Biocheck, #CD100, lot RF22 for *Salmonella* and NDV, lot RF19 for IBV). According to manufacturer instructions, antibody titers were calculated using Eq. (1).

(1)

$$\log_{10}(titer) = 1.13 \times \log_{10}\left(\frac{Mean_{test\ sample} - Mean_{negative\ control}}{Mean_{positive\ control} - Mean_{negative\ control}}\right) + 3.156$$

Data analysis

Flow cytometric data were processed with FlowJo software (Tree Star, V.10.10.0, USA). Statistical analyses were carried out with either GraphPad Prism (V.10.5.0) or R Studio (V4.4.2). All data are shown as mean ± SEM, and either Mann-Whitney, Kruskal-Wallis test, or One-way ANOVA was applied (detailed in the figure legends). Statistical significance was set at $p \leq 0.05$.

Data availability

The analyzed data are available within the manuscript. All raw data obtained from our experiments are available from the corresponding author on reasonable request.

Declarations

Competing interest

This study was designed and conducted by Phileo By Lesaffre. All authors were employees of Phileo By Lesaffre or Lesaffre International.

Funding

declaration

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Author Contribution

J.S. and C.M. designed the experiment, E.B., C.M., L.V. and J.S. conducted the experiments, E.B., L.P. and J.S. analysed the results. E.B. drafted the manuscript and prepared the figures and tables, E.B., L.P. and J.S. revised the paper, L.P. and J.,S. supervised the project. All authors reviewed the manuscript.

Data Availability

The analyzed data are available within the manuscript. All raw data obtained from our experiments are available from the corresponding author on reasonable request.

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Figures

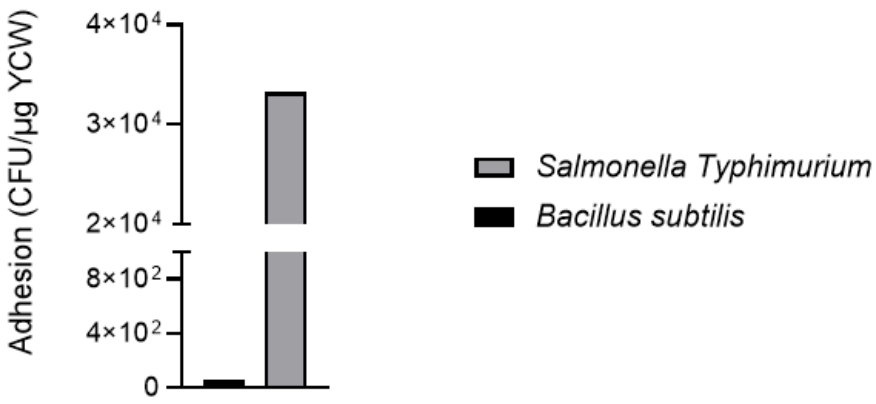


Figure 1

Adhesion of Salmonella Typhimurium to YCW product. 1µg of yeast cell wall bind 3 x 10^4 CFU of Salmonella. Binding control consists of BSA and Salmonella without YCW including all the steps and determines non-specific binding sites, negative binding control consists of Bacillus subtilis and YCW.

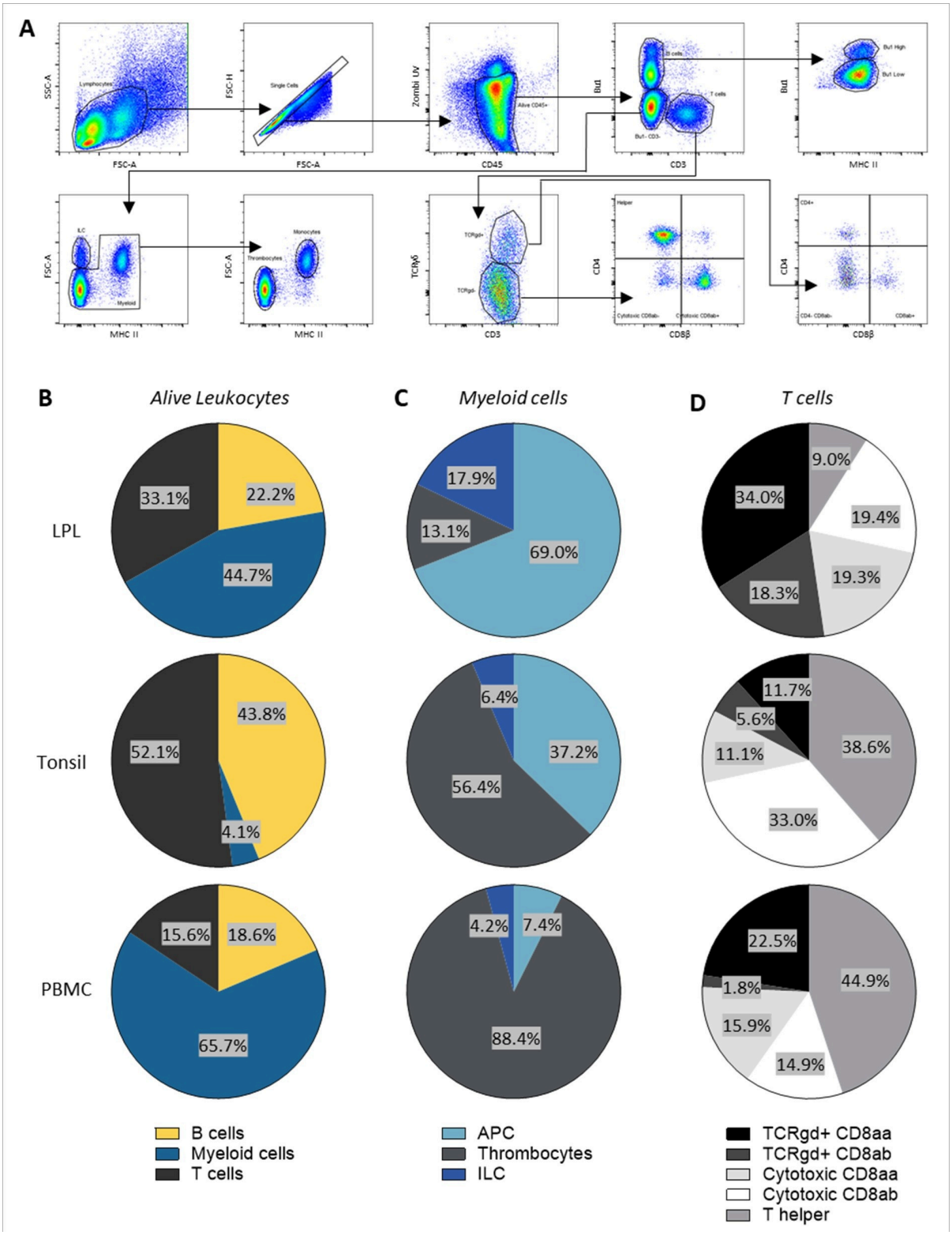


Figure 2

Immunophenotyping of 12-week-old vaccinated chickens. A. Gating strategy used to identify the different leukocyte populations. B. Leukocytes distribution between the B, T, and myeloid lineages in the lamina propria, the blood, or the tonsils. C. Leukocytes distribution

inside the myeloid compartment in the lamina propria, the blood, or the tonsils. D. Leukocytes distribution between the T-cells' subpopulations in the lamina propria, the blood, or the tonsils by a strong predominance of the myeloid compartment, which accounted for 66% of the total leukocytes (Figure 1B).

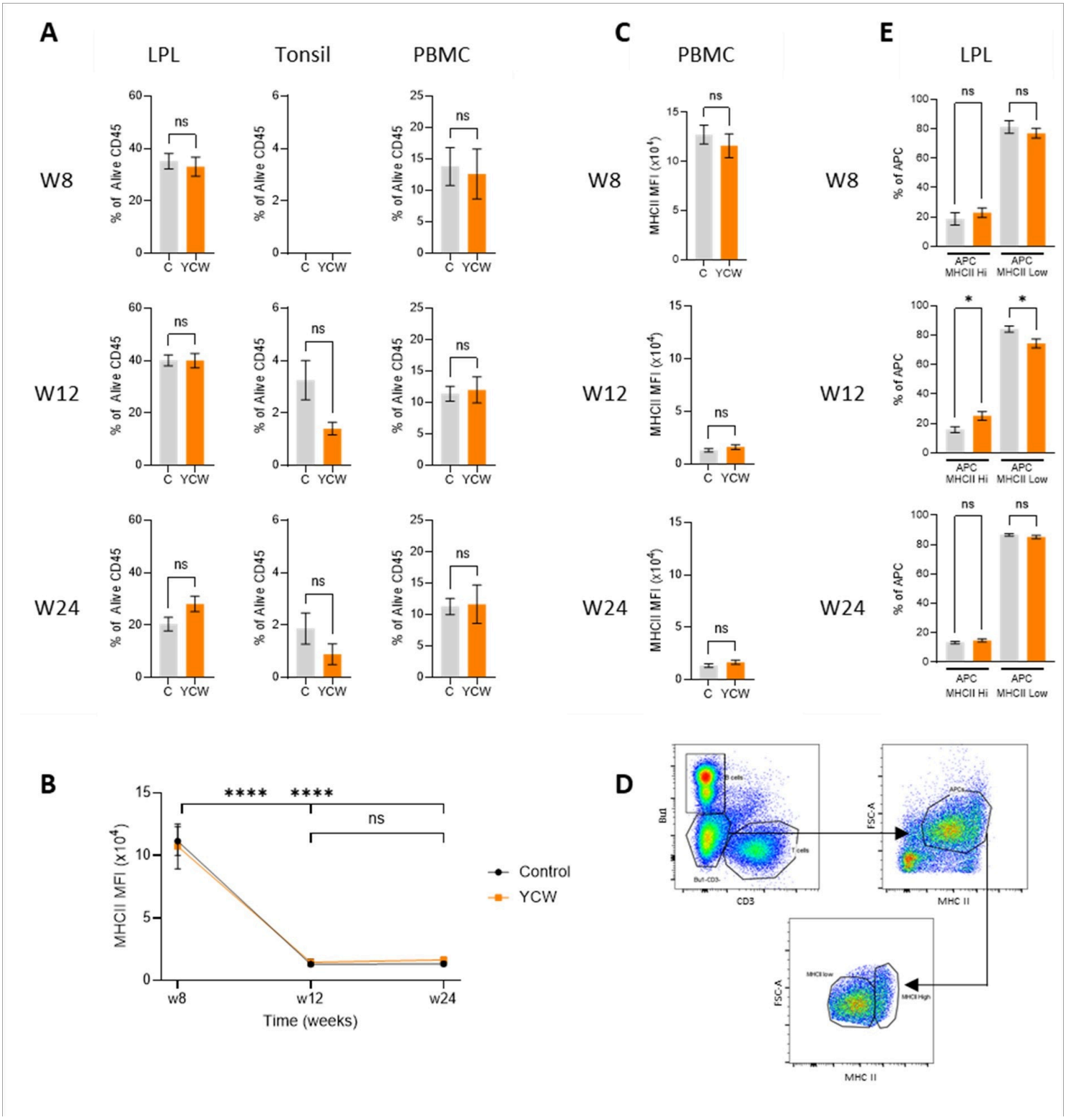


Figure 3

Characterization of the myeloid compartment among YCW-fed animals. A. Myeloid cells frequency, gated for viable single leukocytes $CD45^{+}/Bu1^{-}/CD3^{-}$. B. Evolution of the MHC II MFI calculated by geometric mean over time in the blood APCs. C. MHC II MFI of blood APCs depending on the treatment. D. FlowJo image of the APCs high and low groups. E. Distribution of the Lamina propria APCs based

on their MHC II expression. Data are presented as means +/- SEM (N=5-10). Mann-Whitney test (A, C) or One-way ANOVA (B, E) was applied for statistical analysis (*p ≤0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

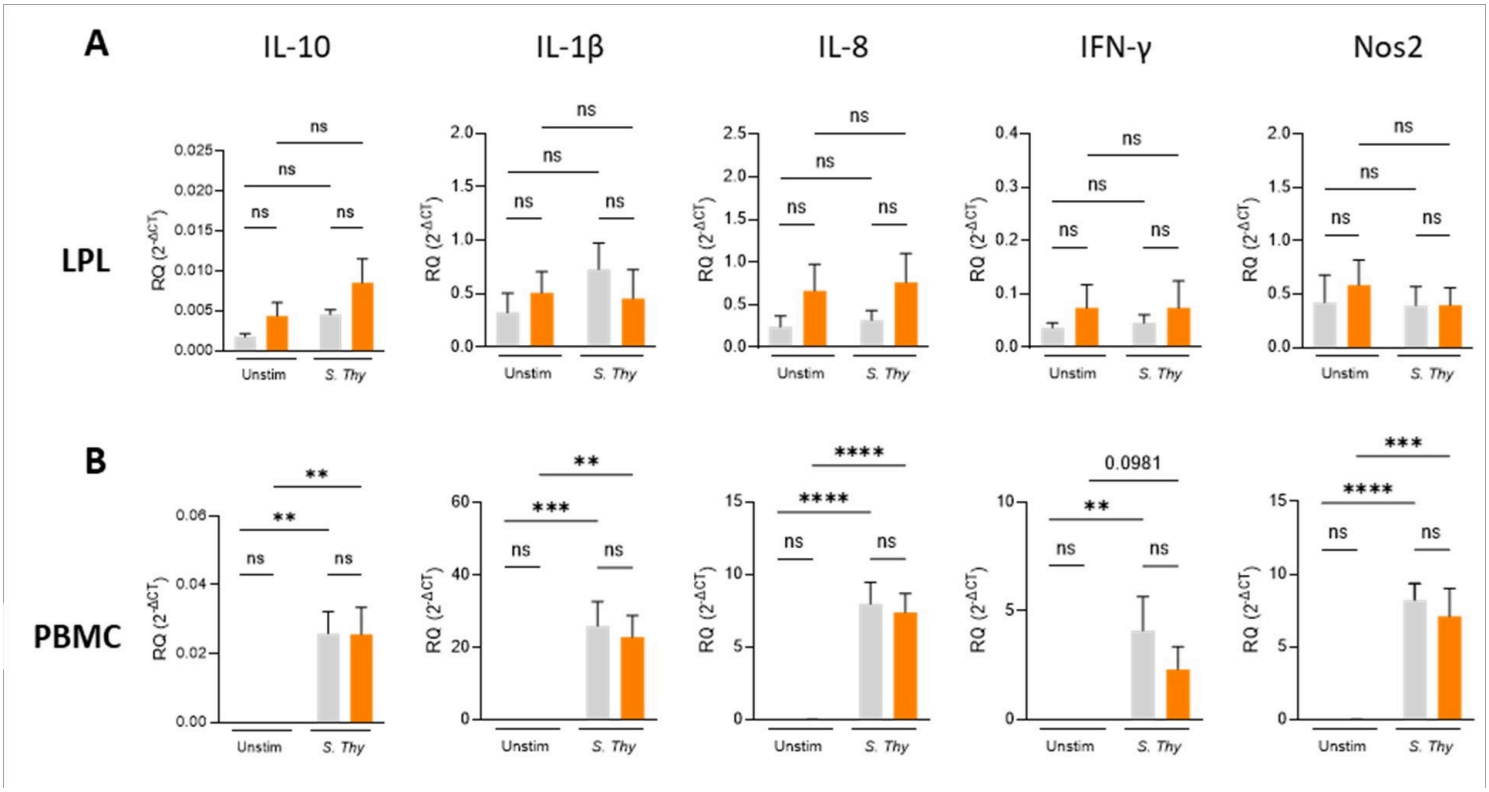


Figure 4

Cytokine production upon in vitro stimulation. Leukocytes were stimulated with S. Typhimurium antigen for 6 hours, and cytokines' mRNA were quantified by Taqman qPCR in both the lamina propria (A) and the blood(B) at 24 weeks for control (gray) and YCW-treated (orange) animals. Data are presented as means +/- SEM (N=6). 2-way ANOVA was applied, followed by post hoc test for statistical analysis (*p ≤0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

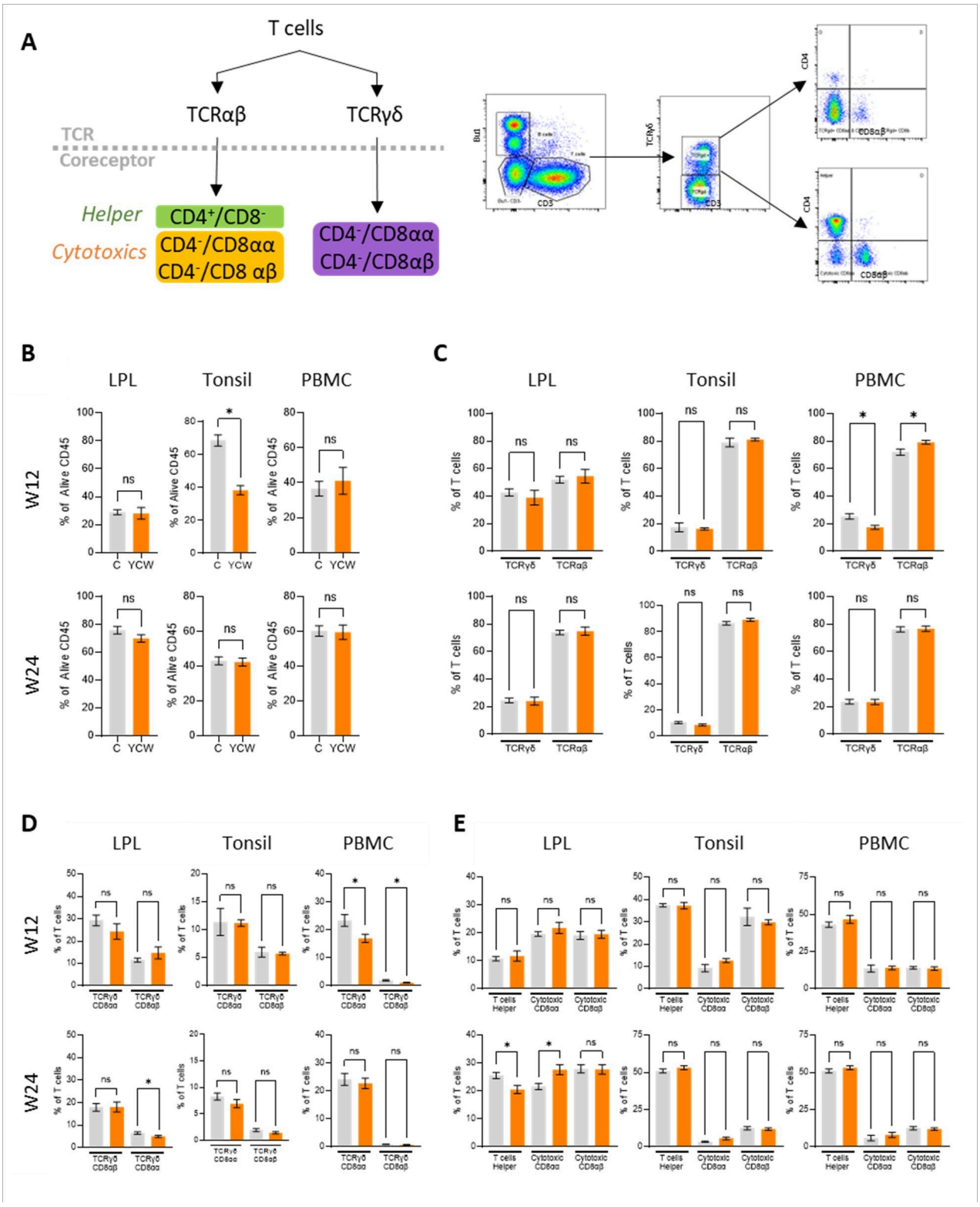


Figure 5

Characterization of the T cell subpopulation. A. Classification of T cells based on their TCR and coreceptor (left) and representative FlowJo images (right). B. T cell frequency, cells gated for viable single leukocytes CD45$^+$/Bu1$^-$/ CD3$^+$. C. Distribution of the T cells into αβ or γδ TCR. D. Coreceptors frequency among TCR γδ$^+$ T cells. E. Coreceptors frequency among TCRαβ$^+$ T cells. Data are presented as means +/- SEM (N=5-10). Mann-Whitney test was applied for statistical analysis (*p ≤0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

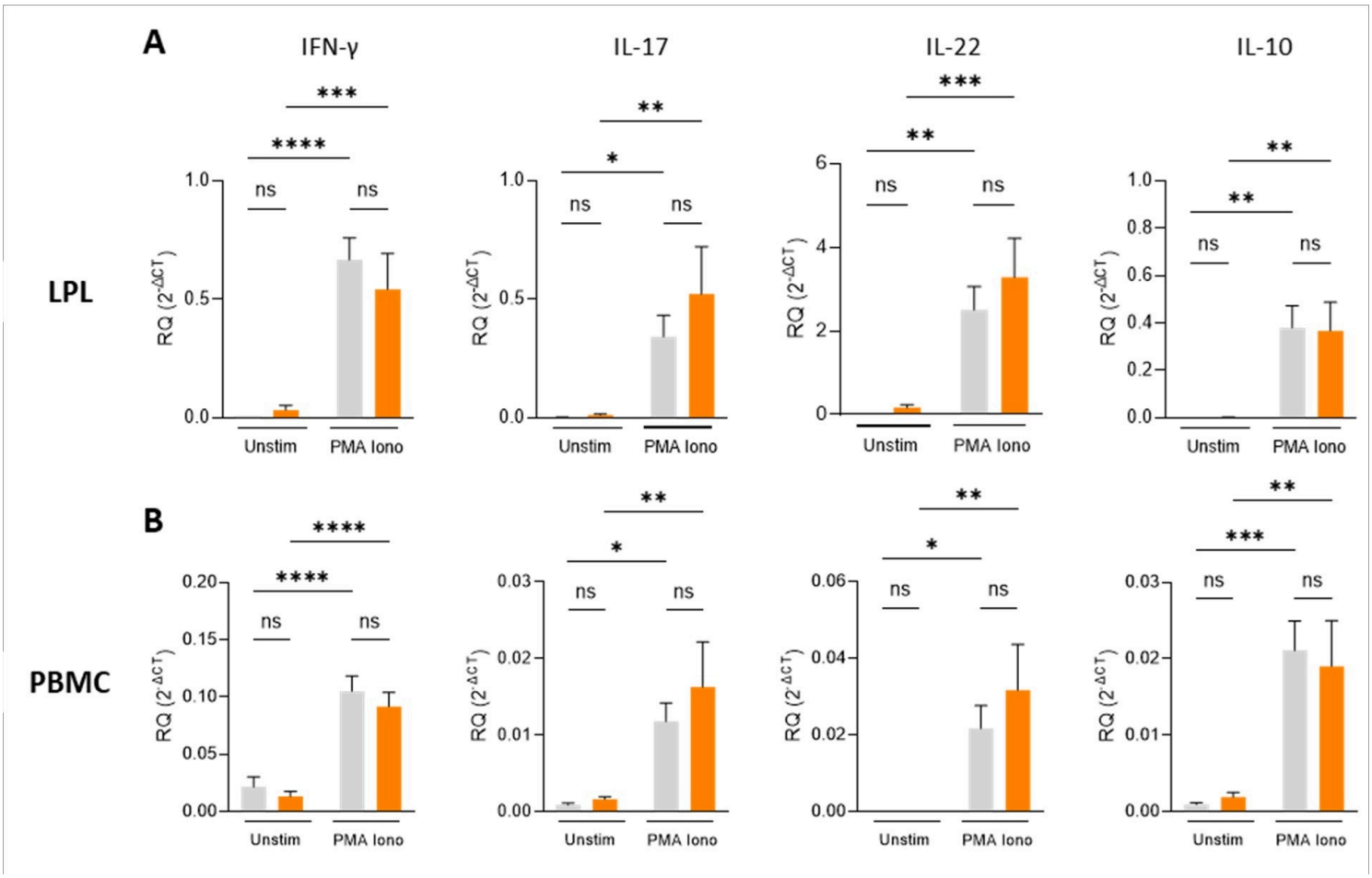


Figure 6

Cytokine production upon in vitro activation. Leukocytes were activated with PMA and ionomycin for 3 hours, and cytokines' mRNA were quantified by Taqman qPCR in both the lamina propria (A) and the blood (B) at 24 weeks for control (gray) and YCW-treated (orange) animals. Data are presented as means +/- SEM (N=6). 2-way ANOVA was applied, followed by post hoc test for statistical analysis (*p ≤0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

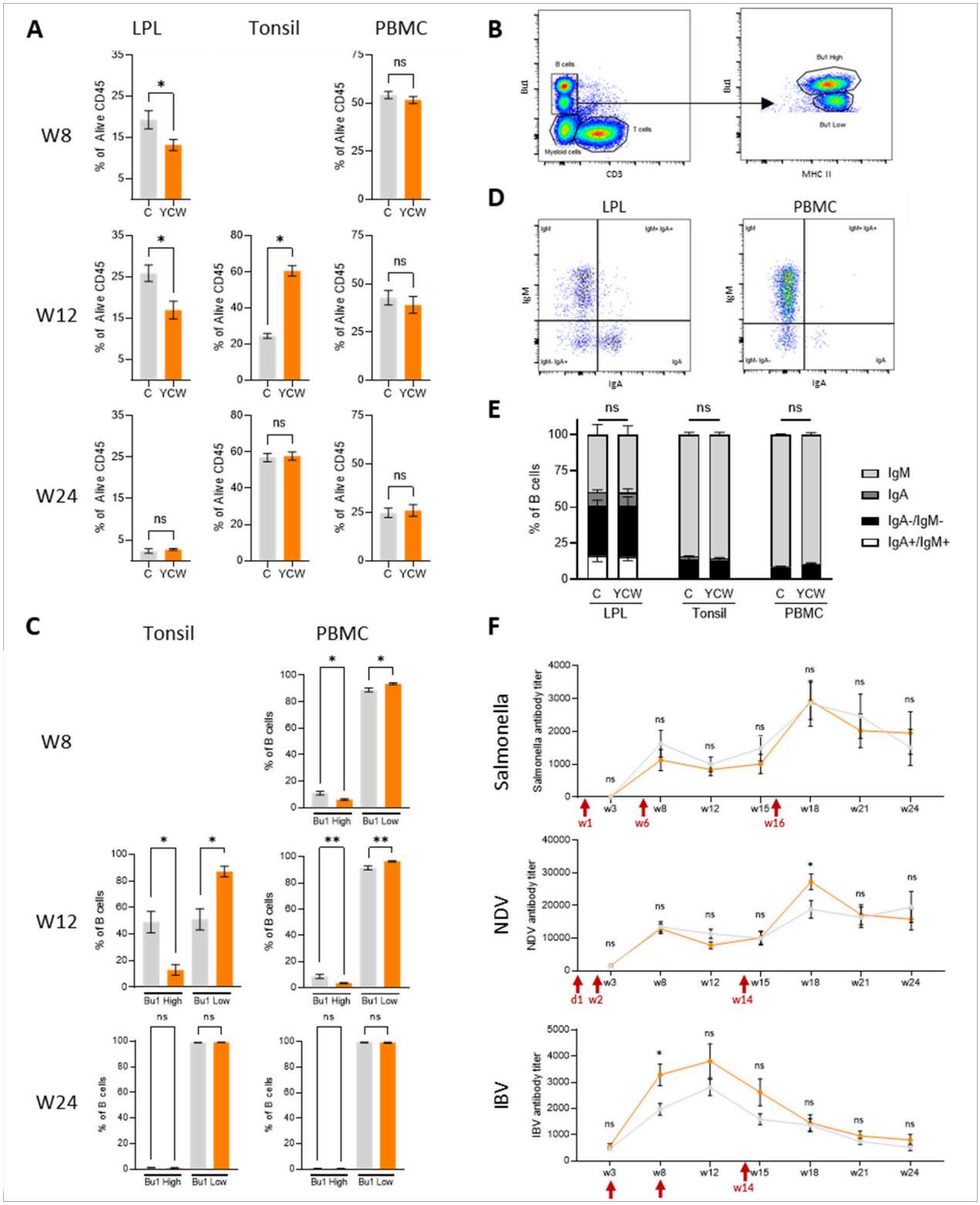


Figure 7

Effect of a YCW diet on the humoral response. A. B cells frequency, gated for viable single leukocytes $CD45^{+}/Bu1^{+}/CD3^{-}$. B. FlowJo image of the B-cells' two maturation levels, based on Bu1 high or low expression. C. B-cells maturation levels based on their Bu1 expression. D. FlowJo image of B-cells' IgA and IgM immunoglobulins in the lamina propria (left) and the blood (right). E. Frequency of the isotypes A and M among the B cells' surface immunoglobulins at 24 weeks. F. ELISA-based quantification of plasma circulating IgG antibodies against Salmonella, NDV, and IBV. The vaccines' shots and boosters are represented by the red arrows. Data are presented as means +/- SEM (N=5-10). Mann-Whitney test (A-C) or Two-way ANOVA (D-E) was applied for statistical analysis (*p ≤0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

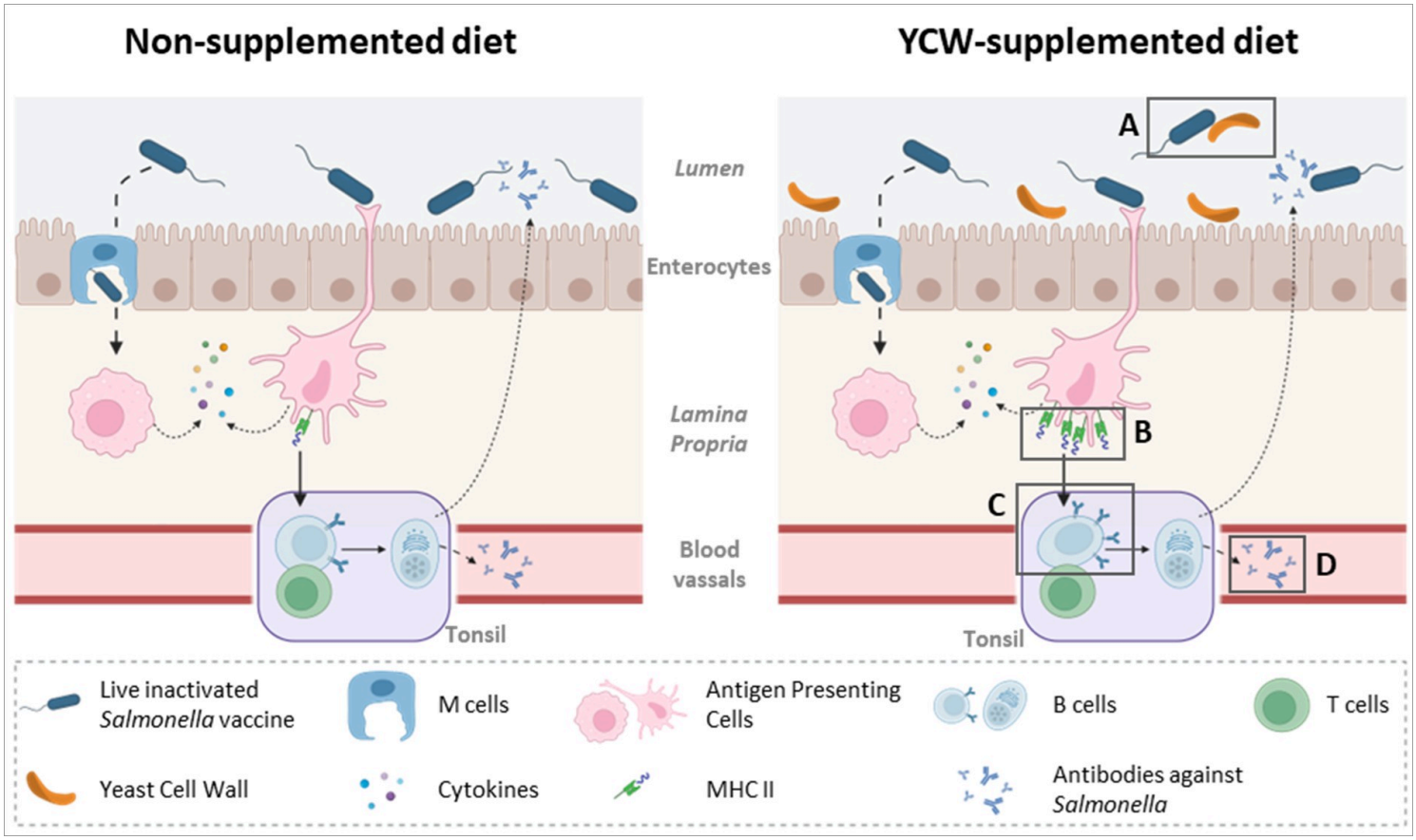


Figure 8

Chicken immune response to oral Salmonella vaccine with (right) or without (left) a YCW-supplemented diet. Only the principal mechanisms and elucidated differences were represented. A. Binding between the YCW and the live attenuated Salmonella vaccine. B. Upregulation of the MHC II by the APCs. C. Increased B-cell maturation. D. Secretion of Salmonella antibodies remains unchanged. Created with Biorender.

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