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Seaweed-derived extracts and their combinations display immunomodulatory activity in a porcine cell culture model

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Abstract

Antimicrobial resistance is a global concern affecting humans, animals, and the environment. Therefore, livestock production is exploring seaweed-based extracts as antibiotic alternatives due to their potential bioactive compounds and immunoregulatory properties. This study aimed to evaluate the *in vitro* immunomodulatory effect of three seaweed extracts: *Ascophyllum nodosum* (AN), *Ulva lactuca* (UL), *Palmaria palmata* (PP), and their 1:1 combinations (ANUL, ANPP, ULPP) on porcine alveolar macrophages (porcine AMs). Briefly, macrophages were seeded at 1 × 10^6 cells/mL and treated with seaweed extracts and challenged with lipopolysaccharide (LPS). The experimental design was a 2 (0 or 1 μg/mL of LPS) × 5 (five doses of each seaweed extract and

their combinations). Cell viability was determined using the MTT assay. The results demonstrated that LPS challenge with certain doses of tested seaweeds decreased ($p < 0.05$) the secretion of tumor necrosis factor-α (TNF-α), interleukin 1-β (IL-1β), and transforming growth factor β1 (TGF-β1). Particularly, a dose-response decrease in TNF-α production was observed for ULPP ($p < 0.05$). PP treatment significantly ($p < 0.05$) suppressed IL-1β secretion at 100 and 200 µg/mL doses, while AN and ULPP (only in doses 100 and 200 µg/mL) significantly ($p < 0.05$) reduced TGF-β1 secretion from LPS-challenged porcine AMs. These results highlighted that seaweed extracts, particularly from PP, and ULPP with their *in vitro* immunomodulatory effects, may be valuable candidates for managing inflammation and encourage further *in vivo* research to confirm their efficacy.

1 Introduction

The increasing demand for safe food raises important questions about sustainable agriculture through the One Health lens, which emphasizes the interconnected health of humans, animals, and the environment [1]. A key challenge within the One Health concept is to curb antibiotic use in both humans and livestock, given the rapid spread of antibiotic resistance [2,3]. In pig production, weaning represents a critical phase during which piglets experience stressors that affect both welfare and growth. Additionally, oxidative stress both triggers and amplifies the inflammatory process, while infections can further exacerbate inflammation. Consequently, various nutritional and management strategies have been tested to support immune responses during infection and to reduce inflammation. In the past, antibiotics were used as growth-promoting agents. However, due to concerns of antimicrobial resistance, the EU banned in-feed antibiotics as growth-promoting agents [4,5] and restricted mass medication during infections [2]. Consequently, bioactive functional feed ingredients, functional products, and phytochemicals are recommended as alternatives to enhance pig welfare and to cope with inflammation [6,7].

Therefore, research is increasingly focused on natural, sustainable molecules to enhance swine health and performance. Seaweed ingredients, and products are thus emerging as a promising source of bioactive compounds [8,9]. Although macroalgae are commercially available as the EU-registered feed materials for use in all animal species [10], their nutritional composition and single bioactive properties are highly heterogeneous. Moreover, no harmonized processing criteria

currently exist to ensure consistency or to define their potential functional activities. Although, chemical composition of seaweeds varies across among the phyla, they are nutritionally rich in proteins [11,12], minerals [13,14], carbohydrates [15,16], and display notable bioactive potential [17,18]. Consistent with this, *in vitro* studies have disclosed that algal extracts and algal-derived compounds possess immunomodulatory properties by influencing cytokine production, suggesting anti-inflammatory effects [19,20]. Accordingly, *Ascophyllum nodosum* (AN, brown), *Palmaria palmata* (PP, red), and *Ulva lactuca* (UL, green) were selected as geographically widespread, aquaculture-farmed, and commercially available representative of three major seaweed phyla. Notably, AN is comprised of polyphenols and phlorotannins with anti-inflammatory potential, including possible inhibition of TRL/NF-κB signaling and cytokine modulation [21–23]. PP with its high protein and vitamin content, may influence insulin response in pigs, and its flavonoids scontent may have potent health-promoting activity [24]. UL is valuable protein source for monogastric diets [25] and has been shown to stimulate cytokine-related gene expression in IPEC-J2 cells [26,27]. Our previous study revealed that UL exhibited the highest content of bioactive compounds, including polyphenols, flavonoids, phlorotannins, and long-chain fatty acids, thus demonstrated their potential as anti-inflammatory agents [28,29].

Moreover, to our knowledge, research on the potentially complementary (multiple effects) and synergistic effects (enhanced activity beyond individual extracts) of combined seaweed extracts is limited [30]. Beyond the activity of the individual extract, our recent research has demonstrated that mixed seaweed extracts exert immunomodulatory effects in porcine alveolar macrophages (porcine AMs) [29]. Among the numerous seaweed species, AN, PP, UL were intentionally selected considering their extensive range of bioactive compounds and distinct chemical compositions, which may promote the potentially synergistic effects. In addition, their widespread geographical distribution further enhances their applicability in the field [17]. Therefore, these extracts in a 1:1 ratio were combined to investigate their possible combined immunomodulatory effects.

Furthermore, porcine AMs are professional phagocytes residing in lung tissue that constitute a first line of immune defense and are key regulators of cytokines production [31,32]. Lipopolysaccharide (LPS) challenges stimulate the release of pro-inflammatory cytokines from these cells [33,34], identifying as an important model for studying innate immune activation. Investigating the effects of seaweed extracts on porcine AMs allows for the assessment of potential immunomodulatory

activities, providing insights into systemic inflammation [35]. Collectively, this underscores the value of porcine AMs as a physiologically relevant and well-established model for assessing the immunomodulatory and anti-inflammatory actions of natural extracts. Moreover, the effects of seaweed on humoral and cell-mediated immunity, particularly in weaned piglets under disease challenge conditions, are not yet fully understood, also highlighting the significance of this model.

Therefore, the novelty of this study resides in the use of extracts derived from commercially available whole-seaweed products rather than purified bioactive compounds. Using this approach, the immunomodulatory potential of three commercially available distinct macroalgal species was evaluated. Moreover, this research evaluated these extracts, both individually and in 1:1 combinations, thereby providing new evidence on their potential synergistic immunomodulatory effects in LPS-stimulated porcine alveolar macrophages, supporting their application as sustainable functional feed ingredients. Based on this framework, the hypothesis of this research was that seaweed-based extracts will exhibit immunomodulatory properties by reducing the inflammatory response in porcine AMs challenged with LPS. Thus, the aim of this research was to estimate the immunomodulatory activity of different extracts prepared from commercially available whole-seaweed products (AN, PP, and UL) and their 1:1 extract mixes (*A. nodosum* and *P. palmata* ANPP, *A. nodosum* and *U. lactuca* ANUL, *U. lactuca* and *P. palmata* ULPP) as new phytochemical sources via measuring the *in vitro* anti-inflammatory properties in porcine AMs after LPS challenge.

2 Results

2.1 Yield and cytotoxicity of seaweed extracts

The extraction yields for seaweed extracts were as follows: 36.75% ± 2.86 for AN; 33.75% ± 2.04 for UL; 33.75% ± 2.28 for PP; 32.25% ± 2.59 for ANUL; 34.5% ± 2.06 for ANPP; 31.75% ± 2.28 for ULPP). Cell viability of porcine AMs was assessed. In this study, no cytotoxic effects were detected at the highest concentration of any of the seaweed extracts or their combinations. The mean of each group, from all wells (n=6), LPS-challenged and unchallenged controls, and those exposed to seaweed extracts, exhibited ≥ 90% cell viability (where ≤70% cell viability is considered the minimum level indicating a significant cytotoxic effect). All treatments resulted in no statistically significant differences in cell viability, except for PP in dose (Tab. 1). However,

non-significant cell proliferation was observed in porcine AMs treated with some doses of LPS and NO LPS-challenged seaweed extracts (Tab. 1).

Tab. 1. MTT cell viability (%) of porcine AMs with or without different seaweed extracts, with significant differences ($p < 0.05$) among treatments in the presence or absence of LPS. Data are presented as means ± standard error of the mean (SEM).

2.2 Immunomodulatory properties of seaweed extracts

The LPS challenge triggered ($p < 0.05$) the production of pro-inflammatory cytokines tumor necrosis factor-α (TNF-α, Fig. 1A–F), interleukin 1-β (IL-1β; Fig. 2A–F) and reduced the production of the multifunctional cytokine transforming growth factor β1 (TGF-β1; Fig. 3A–F). Under LPS stimulation, certain seaweed extracts and their combinations significantly modulated TNF-α production in a dose-dependent manner (Fig. 1). The lowest dose of AN (25 μg/mL) reduced TNF-α production compared with the control. Additionally, ANPP significantly decreased TNF-α production at the lowest extract doses (25 μg/mL and 50 μg/mL; Fig. 1E). Conversely, UL significantly reduced TNF-α production only at the highest dose (200 μg/mL; Fig. 1B). A decreasing significant difference in cytokine production was also observed for two doses of PP (50 and 200 μg/mL; Fig. 1C) and for all doses of ULPP (Fig. 1F). A significant increase in TNF-α production was observed with the ANUL extract (Fig. 1D). At the highest concentration (200 μg/mL), the greatest reductions in cytokine secretion relative to the LPS control were observed in treatment with UL, PP and ULPP (81.70%, 76.2%, and 82.20%, respectively).

Furthermore, in the presence of an LPS challenge, seaweed extracts and their combinations (except AN) affected IL-1β production in a dose-dependent manner (Fig. 2A–F). Treatment with PP led to a reduction of IL-1β at the two lowest doses, and significantly inhibited ($p < 0.05$) IL-1β secretion starting at 100 μg/mL dose in LPS-challenged porcine AMs. Moreover, treatment with ANUL tended to decrease the production in a dose-dependent manner and significantly inhibited the secretion at the highest dose (200 μg/mL; Fig. 2C, and D). Additionally, one dose of ULPP (25 μg/mL) significantly ($p < 0.05$) reduced IL-1β production compared to the control (Fig. 2F). At 200 μg/mL, PP and ANUL reduced cytokine secretion by 75.10% and 54.10%, respectively, versus the LPS control.

For the multifunctional cytokine (TGF-β1), in the presence of an LPS challenge, seaweed extracts and their combinations affected TGF-β1 production (Fig. 3A–F). Specifically, AN and ULPP

significantly decreased ($p < 0.05$) the secretion of TGF-β1 from porcine AMs in the presence of LPS, where ULPP significantly reduced TGF-β1 secretion at higher concentrations (Fig. 3A and F). Additionally, UL at two tested concentrations (25 and 100 µg/mL) significantly ($p < 0.05$) reduced TGF-β1 production compared to the control (Fig. 3B). In contrast, PP exhibited a significant dose-response effect in regulating this cytokine's production (Fig. 3C). At 200 µg/mL, PP and ULPP produced the greatest reductions in cytokine secretion relative to the LPS control (80.50% and 69.40%, respectively).

3 Discussion

The objective of this study was to assess the *in vitro* immunomodulatory activity of three commercially available seaweed extracts (AN, UL, PP) and their 1:1 combinations (n=6) by evaluating their immunomodulatory properties, as our previous results revealed their strong antibacterial and antioxidant properties with demonstration of their high polyphenol content [28,29]. This involved using macrophage cells and testing various doses of the seaweed extracts and their mixtures under LPS challenge. This research demonstrated that selected seaweed extracts modulate the production of cytokines in macrophages. Our study revealed the potential of certain tested seaweed extracts (especially from PP) and their combinations to reduce LPS-induced production of pro-inflammatory cytokines in porcine AMs. Notably, *P. palmata* and its combination with *U. lactuca* (ULPP) emerged as more powerful candidates as they were able to block the secretion of all measured pro-inflammatory cytokines (TNF-α and IL-1β) and the multifunctional cytokine (TGF-β1). Thus these extracts highlighted their immunomodulatory potential and prioritize of PP and ULPP for further *in vivo* research.

Further, no cytotoxic effects were detected at the highest tested doses of any seaweed extract. The MTT assay results confirmed that the immunomodulatory properties of the seaweed extracts were not due to direct cytotoxicity. Interestingly, some extracts in this study promoted the proliferation of porcine AMs, as the MTT measures the reduction of tetrazolium salt by mitochondrial dehydrogenase, and thus values exceeding 100% of the control reflect an increase in cell number due to proliferation or an enhancement of mitochondrial metabolic activity, rather than an experimental artifact [36]. Such MTT responses have been reported with various bioactive compounds. Kovanda et al. [37] detected that short-chain fatty acids significantly enhanced the

proliferation of intestinal epithelial cells. Similarly, Donohoe et al. [38] demonstrated that butyrate-induced proliferation in HCT116 cells is linked to increased mitochondrial oxidative phosphorylation. In the context of seaweed-derived compounds, polysaccharides such as fucoidan, and β-glucans may biostimulate, immunostimulate macrophages, and upregulate their mitochondrial activity [39]. Furthermore, the long-chain fatty acids (α-linolenic and oleic acid) and polyphenols identified in our previous study may enhance mitochondrial biogenesis, thus explaining the MTT values observed in treatment groups in the present study [29]. Although the MTT is widely used to assess viability and proliferation, further analyses, such as flow cytometry, are needed to better understand mitochondrial function and cell cycle progression.

Pro-inflammatory cytokines such as TNF-α and IL-1β are primarily produced by activated macrophages, and play a crucial role in the initiation of inflammatory responses and regulate the host immune response. However, the overproduction of these pro-inflammatory cytokines causes inflammatory diseases [33]. The current study demonstrated that in LPS stimulation, certain seaweed extracts, especially from red seaweed, *P. palmata* (PP) decreased the production and secretion of TNF-α and IL-1β from macrophages, especially at the highest dose, consistent with previous research [20,40–43]. Another study reported that the potent activity of ascophyllan, extracted from *A. nodosum*, promoted cytokine induction in mouse RAW 264.7 cells, in a concentration-dependent manner [44]. Our study also led to a decrease in TNF-α production in macrophages treated with low-dose AN extract, however higher doses increased TNF-α concentration. Taken together, these findings suggest that the increase in TNF-α observed at higher AN extract doses may reflect a hormetic response, whereby low concentrations exert anti-inflammatory effects while higher concentrations activate macrophage signalling pathways [45]. Further, in line with our study, where we observed a significant difference in TNF-α reduction at the highest dose of UL, research by Wang et al. [46] reported that *U. lactuca* extracts inhibited the release of TNF-α, and upregulated the expression of Th2 cytokines such as interleukin 10 (IL-10) in RAW 264.7 cells with pro-inflammatory challenge. Further, polyphenol-rich components extracted from *U. lactuca* demonstrated a suppressive effect on mRNA expression of TNF-α, and IL-6 in activated macrophages [47]. Regarding IL-1β detection, another study showed that *P. palmata* did not inhibit the production of IL-1. However, significantly inhibited ($p < 0.05$) the production of other inflammatory inflation marker cytokine, interleukin (IL)-6 in LPS-challenged human THP-1 macrophages [20]. A different study demonstrated that the production of TNF-α significantly

decreased with increasing concentrations of digested *P. palmata* water extract, revealing suppressive effects at 50 µg/mL in LPS-challenged RAW 264.7 cells. Treatment (LPS-stimulated RAW 264.7 cells) with 100 µg/mL of thermolysin-digested water extract of *P. palmata* reduced the production of TNF-α to 60.60%. Further, the addition of 250 µg/mL showed the almost maximum suppressive effect on the secretion of TNF-α[48].

Among the cytokines involved in the inflammatory process, TGF-β performs a vital role as a pleiotropic cytokine in immunoregulation. Depending on the cellular and environmental context, TGF-β can exert distinct anti-inflammatory, immunosuppressive, and pro-fibrotic functions [49]. In the context of our study, its anti-inflammatory and immunosuppressive activity is particularly relevant, as TGF-β1 (an isoform of TGF-β) can inhibit the differentiation and proliferation of T and B lymphocytes and deactivate monocytes and macrophages. Thereby dampening excessive inflammatory responses [50]. In our study, only some seaweed extracts ($p < 0.05$) reduced TGF-β1 secretion from porcine AMs upon LPS stimulation, and not all extracts were effective at all tested doses. Specifically, all doses of AN, certain doses of UL (especially at 25, 100 µg/mL), and the ULPP mix extracts at doses of 100 and 200 µg/mL demonstrated a reduction in TGF-β1 secretion. The underlying mechanism behind these observations requires further clarification, as existing findings do not fully align with our data regarding the production of TGF-β cytokine. The study by Wang et al. [46] reported that *U. lactuca* extracts upregulated the expression of TGF-β1 in RAW 264.7 cells in the presence of a pro-inflammatory challenge. Furthermore, no *in vitro* studies have tested *P. palmata* for TGF-β secretion. However, the discrepancy between our findings and the existing literature may be attributed to differences in seaweed species, extract composition, and the cellular models used in these experiments.

Overall, our findings indicate that the tested seaweeds, particularly the red seaweed *P. palmata,* may exert anti-inflammatory activity through the modulation of TGF-β1 production in porcine AMs. The consistent reduction in TGF-β1 observed in the first two doses of PP extract and all doses of ULPP extracts suggests that its bioactive components might interact with early steps of the LPS-induced inflammatory cascade, leading to a restrained activation of downstream cytokine signaling. We suppose, based on literature findings, that these extracts may influence pathways involved in macrophage activation, such as the TLR4-dependent response or the regulation of Smad-mediated transcription, ultimately modulating cytokine secretion profiles [51,52]. In contrast,

the effects of AN and UL extracts were more heterogeneous. The reduction in TGF-β1 at selected concentrations indicates that their modulatory activity may be contingent on achieving specific intracellular concentrations of active metabolites. These findings point to the presence of dose-sensitive biological activities that vary across seaweed species and extract compositions. This observation is in line with previous research indicating that seaweed-derived polysaccharides can modulate immune responses [53]. Additionally, the findings highlight the importance of determining the optimal dosage to maximize efficacy, as dosage played a critical role in cytokine determination in selected seaweed extracts. This is essential to achieving therapeutic benefits while minimizing potential adverse effects.

Furthermore, this study evaluated the potentially synergistic and complementary properties of bioactivities derived from mixed seaweed extracts (1:1 combinations). The aim was to characterize their interactions for therapeutic efficacy and to lower the minimal effective dosage required to achieve combined effects. The synergistic effect of two agents is more potent than the effect of either agent alone on a specific activity, whereas the complementary action of the two agents combines two or more distinct effects [30]. Our results suggest that *P. palmata* may show a potentially important role in reinforcing the efficacy of other tested seaweeds. Notably, its combination with *U. lactuca* resulted (ULPP) in a significant reduction in the production of pro-inflammatory cytokines TNF-α and IL-1β (only at concentrations of 25 µg/mL). This finding indicates that PP may contribute to lowering the minimal effective dose against infection. However, further studies are required to clarify the mechanisms of action, as well as to validate these findings given the limited literature on this specific interaction.

Although the precise modes of action underlying the anti-inflammatory effects of plant- and seaweed-based extracts remain unclear, the significant modulation of TNF-α, IL-1β, and TGF-β1 secretion observed in the present study suggests potential involvement of inflammatory signaling pathways. Based on previous literature, these effects may be partly mediated by the inhibition of the TLR4/NF-κB axis and MAPK cascades, which represent central regulators of LPS-induced inflammatory responses in macrophages [54,55]. Although these pathways were not directly investigated in the present study, according to the literature findings, NF-κB plays a central role in the activation, differentiation, and effector function of inflammatory responses. Thus its inhibition has been proposed as a key mechanism underlying the anti-inflammatory effects of seaweed-

derived bioactive compounds [55,56]. However, in the absence of direct mechanistic assays, such interpretations remain speculative. These compounds present in the tested seaweed extracts are hypothesized to interfere with these signaling cascades at multiple levels, in a compound- and species-specific manner. For example, AN, phlorotannins and polyphenols, the most abundant bioactive classes in this macroalga [28], have been reported to inhibit TLR4-dependent NF-κB activation and suppressed TNF-α transcription in activated macrophages [57,58]. UL, sulfated polysaccharides and polyphenol-rich fractions, suppressed mRNA expression of TNF-α, and IL-6 in LPS-activated macrophages, through inhibition of NF-κB nuclear translocation [59]. In PP, flavonoids and polyunsaturated fatty acids, particularly α-linolenic acid identified in our previous metabolomic characterization [29], may modulate eicosanoid signaling and attenuate arachidonic acid-derived pro-inflammatory mediator production. Thereby reducing both TNF-α and IL-1β secretion [60]. The consistent and broad-spectrum suppression of pro-inflammatory cytokines observed for PP across multiple doses indicates a multi-target anti-inflammatory effect. Although the exact molecular basis remains to be further clarified.

The anti-inflammatory properties of bioactive compounds in seaweeds are recognized for their ability to modify inflammation and oxidative stress through various signaling pathways. This highlights their potential to mitigate inflammation in several diseases [40,61]. Indeed, various extracts from microalgae and macroalgae have been shown to moderate the release of inflammatory mediators while exhibiting low cytotoxicity towards cells [62,63]. This is consistent with the absence of cytotoxic effects observed in the present study. In this context, it should be acknowledged that the present study focused on the cytokine secretion at the protein level, and did not directly assess the underlying transcriptional or signaling mechanisms. Nevertheless, in our recently published related paper [29], the same seaweed extracts were evaluated at the gene expression level in porcine AMs. That study reported a significant downregulation of IL-1β, and TNF-α and a modulation of TGF-β1 expression, findings fully consistent with the protein-level results in this research. Therefore, these complementary data suggest that seaweed-derived bioactive compounds can modulate inflammatory responses through mechanisms that may involve transcriptional and post-transcriptional regulations, acting on the LPS–TLR4–NF-κB axis as well as on MAPK and Smad-mediated signaling, as suggested by literature findings. Although further investigations, such as transcriptomics, proteomics, NF-κB/MAPK/TLR signaling analysis, and targeted studies on individual bioactive fractions, are required to fully elucidate the involved pathways. These

approaches are also necessary to determine the relative contribution of each bioactive class to the observed immunomodulatory effects [29].

4 Conclusion

With antibiotics banned as growth promoters, and the restriction of mass medication in swine, interest has increased in bioactive feed ingredients and functional products such as seaweed extracts and phytochemicals to enhance immunity and disease resistance. Seaweeds offer diverse bioactive compounds, yet their specific effects require careful evaluation. The findings of this study emphasized the *in vitro* immunomodulatory properties of selected extracts derived from seaweed extracts from whole commercially available products. Notably, the red seaweed (PP) and its combination (ULPP) extracts had the highest effectiveness in TNF-α, and IL-1β regulation across multiple concentrations (50 and 200 µg/mL; 100 and 200 µg/mL; 25 to 200 µg/mL; and 25 µg/mL, respectively), suggesting their potent immunomodulatory activity. Additionally, the results illustrate the importance of determining the optimal dosage levels to maximize efficacy. Therefore, the tested seaweed extracts may have potential for application as feed ingredients and functional products to reduce the severity of bacterial infection and could play a significant role in the development of novel functional nutritional strategies aimed at decreasing reliance on antibiotic treatment in farming systems. These implications require further *in vivo* verification to determine any therapeutic potential in animal models.

5 Material and methods

5.1 *Materials and experimental design*

The standardized 100% pure lyophilized wild-harvested powders of *A. nodosum* (AN; number: SX 009776) and *U. lactuca* (UL; number: SZ 009874) were commercially sourced from Italfeed Srl (Milan, Italy), while the powder of *P. palmata* (PP; number: 10418) was obtained from Alganex GmbH (Berlin, Germany), in compliance with European safety requirements. Prior to further immunomodulatory analysis, an ethanol-based extraction was performed on the 100% pure seaweed powders, both from various seaweed species and their combination.

To prepare cell culture treatment, seaweed extracts were diluted with sterile RPMI-1640 (Roswell Park Memorial Institute) medium for the cultivation of porcine AMs. Moreover, AN extracts were

initially dissolved in dimethyl sulfoxide (DMSO). Culture media were supplemented with 1% antibiotics (100 mg/mL penicillin and 100 mg/mL streptomycin; Mediatech, Inc., Manassas, VA) and with 5% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). The ultimate concentration of DMSO in the media did not exceed 0.05%. According to the literature, even higher concentrations of DMSO are considered acceptable in small-molecule cell-based assays without affecting normal cellular growth [64]

This experiment contained 6 individual *in vitro* assays for testing 6 seaweed extracts, and all seaweed extracts were evaluated using a factorial design with 5 doses (0, 25, 50, 100, and 200 μg/mL) × 2 LPS levels (0 or 1 μg of LPS/mL) × 6 extract types (AN, PP, UL and their 1:1 combinations). The experiments were conducted in a randomized complete block design, with the blocking factor as donor piglets (randomized complete blocks), resulting in a total of 10 treatments for each seaweed extract. Fifteen donor piglets were used as biological replicates, and each extract was evaluated using PAMs from five independent piglet donors. Further, multiple PAMs wells (n=60) measured within the same donor and treatment combination were considered technical replicates. The experimental treatments included a negative control, which consisted of no seaweed extract and non LPS, and a positive control, which included no seaweed extract but LPS. The seaweed extracts were administered at doses of 0, 25, 50, 100, and 200 μg/mL. These dose concentrations were selected based on previous studies of our team, with plant-derived extracts [65]. The experimental unit was macrophages (cell pool of 3 wells). The LPS was sourced from *Escherichia coli* O111:B4 (Sigma Co., St. Louis, MO, USA).

5.2 *Seaweed biomass extraction*

The biomass of the tested species was extracted using ethanol as the solvent, following the literature methodology [66,67] with some modifications developed by our team [28]. Briefly, seaweed biomass powder was dissolved in 80% ethanol (1:10 ratio), milled with a mortar, and vortexed (3 min). Subsequently, samples were frozen overnight (-20°C) to optimize the extraction procedure, followed by centrifugation (3,000 × g × for 20 min at 4°C). The supernatant was decanted, after which the solid glass beads (3 mm) were added to each sample. The glass bead solution was then homogenized for 30 s × 4.5 RPS (FastPrep-24 classic homogenizer, MP Biomedical, Irvine, CA, USA), and samples were centrifuged again (3,000 × g × for 20 min, at 4°C). Ethanol extraction of

the remaining pellet was repeated three times. Afterward, the extraction solution was evaporated (Rotary Evaporator Strike 300, Steroglass srl, Perugia, Italy) at a temperature not exceeding 50°C. The resulting dried residues were weighed, and the yield was calculated based on the weight of the dry seaweed powder (data presented as mean and standard deviation). Each residue was subsequently resuspended in sterile RPMI-1640 medium (Roswell Park Memorial Institute) to the desired concentrations, then filtered (using 0.22 µm syringe filters) to dissolve any particles, under sterile conditions, for further analysis.

5.3 *In vitro* immunomodulatory assays

5.3.1. Harvesting of porcine alveolar macrophages

Porcine AMs were collected from commercially euthanized animals, in accordance with the 3R principles, and global ethical guidelines for the use of animals in research, from healthy breeding animals intended for the commercial meat-processing industry in compliance with EU directive [68]. No research activity was conducted on living animals. Porcine AMs were directly isolated from healthy trade-intended animals in the meat-processing business, which were euthanized during meat-processing procedures. Furthermore, industrially traded animals prior to euthanasia met established health requirements, exhibited good general health status, and underwent thorough ante-mortem and post-mortem inspections to confirm the absence of clinical signs of infectious diseases. Animals were euthanized at an authorized abattoir in accordance with standard EU animal welfare procedures. Stunning was performed by electronarcosis.

As a result, porcine AM cells were assembled from commercially euthanized Polish Landrace breed piglets (n=15, 50% male and 50% female, for balanced sampling) at 10 weeks and approximately 22–24 kg of body weight. Before the commercially euthanized procedure, piglets were farmed for the food production industry, housed in a room at a constant temperature (21°C) and humidity (60%), and received a basal pre-starter diet and basal diet *ad libitum* formulated according to nutritional requirements for the nursery and weaning phases (Pigwit Starter MPU, Cargill, Poland; PigLead Neonatal Pro, Cargill Poland, accordingly). Each pen was equipped with nipple drinkers with *ad libitum* access to fresh water.

Macrophages were collected from commercially euthanized animals, through bronchoalveolar lavage following the procedures described by Baarsch [69] and our previous research [65]. Briefly, lungs with intact trachea were harvested immediately after euthanizing the piglets post-mortem, in order to preserve cell viability and minimize post-mortem alterations, and 150 mL of ice-cold phosphate-buffered saline (PBS) without calcium or magnesium was infused through the trachea. The lungs were gently massaged for approximately 60 s to facilitate lavage, and the resulting fluid was filtered through a double-layer of sterile gauze into 50 mL conical centrifuge tubes and then pelleted by centrifugation at 400 × g for 10 min (at room temperature). The cell pellets were resuspended in 4 of mL ACK lysing buffer (Thermo Fisher, Massachusetts, USA) to lyse red blood cells, followed by the addition of 5 mL of RPMI-1640 culture medium. Cell viability of live cells was assessed using trypan blue dye exclusion (Sigma-Aldrich Co., St. Louis, MO, USA), and counted with a hemocytometer (Fisher Scientific, Inc., Pittsburgh, PA, USA). The final concentration of the cells was adjusted to 1×10^6 cells/mL with viability exceeding 97%. For this study, the term *porcine alveolar macrophages* was used, as macrophages constitute the predominant cell type isolated from bronchoalveolar lavage fluid [70].

5.3.2. Cell culture

Porcine AMs were cultured at a density of 1×10^6 cells/mL in a cluster of either plates (24-well or 96-well). All plates were incubated overnight at 37°C in a humidified incubator with 5% CO_2, allowing macrophages to adhere to the bottom of the wells. Following incubation, non-adherent cells were removed by washing the wells with pre-warmed RPMI-1640 medium. Adherent macrophages were then treated with various treatments in duplicate, using fresh, pre-warmed RPMI-1640 medium. After a further 24 h incubation, supernatants were collected in duplicate, pooled, and stored at –80°C for subsequent cytokine analysis.

5.3.3. Detection of cell viability

To determine the cytotoxicity of seaweed extracts on porcine AMs, the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT Cell Proliferation Assay Kit) assay was performed (Invitrogen, Vybrant MTT Molecular Probes Inc., Eugene, OR, USA) according to our previously described method [65]. This assay assessed the cellular metabolic activity through a colorimetric reaction catalyzed by mitochondrial enzymes, enabling the determination of the number of viable

cells [71]. The optical density (OD) was measured at 540 nm (Synergy HTX Multi-Mode Microplate Reader, BioTek, Winooski, VT, USA) following a 10 min incubation. The background signal, which corresponds to the absorbance of the plates in the absence of cells, was subtracted from the absorbance values obtained for each sample. The average OD of the negative control was determined and set at 100%. The relative viability of each well was estimated using the formula described below. The percentage of live cells indicates cell viability and proliferation, with a live cell percentage below 50% considered a cytotoxic dose.

$$\%\ \textbf{of cell viability} = \left(\frac{\text{OD treated cells}}{\text{mean OD of negative control}}\right) \times 100$$

5.3.4. Measurements of immunomodulatory cytokines

Protein concentrations of pro-inflammatory cytokines (TNF-α, with a catalog number: PTA00, and IL-1β, with a catalog number: PLB00B) and the multifunctional one (TGF-β1, with a catalog number: DB100C, SB100C, DB100C) using alongside with Sample Activation Kit 1 (catalog number: DY010), in cell culture supernatants were assessed using commercial ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions (Fig. 4). Briefly, standards, controls, and samples were added to the wells (50 µL/each), pre-coated with a monoclonal antibody specific for each cytokine (not disclosed in commercial assay kits). Both the LPS and NO LPS samples were diluted, thus the read concentrations have been multiplied by the dilution factors (for LPS samples: TNF-α, 140, IL1-β and TGF-β1, 9; for NO LPS samples: TNF-α, 20, IL1-β, 7, and TGF-β1, 4). After a 2 h incubation at room temperature, the unbound substances were removed by washing, and a polyclonal antibody conjugated to an enzyme, specific for the target cytokine, was added to the wells. Following another 2 h incubation at room temperature, the wells were washed again to remove unbound antibody-enzyme reagent. Subsequently, a substrate solution was added to the wells, causing color development proportional to the amount of cytokine bound during the initial step. A stop solution (50 µL/well of stop solution with hydrochloric acid) was then added to terminate the reaction, and absorbance was measured at 540 nm (Microplate reader Synergy4, BioTek, Winooski, VT, USA). The concentration of individual cytokines was determined (by BioTek Gen 5 Software) using standard curves (four-parameter logistic (4-PL), with a coefficient of determination: $R^2 > 0.97$). All samples were performed in duplicate.

5.4 Statistical analysis

All data created from various assays were analyzed by ANOVA using the MIXED procedure (SAS 9.4 Inst. Inc., Cary, NC, USA) with appropriate statistical models. Prior to analysis, data distribution was assessed, normality was tested using the Shapiro-Wilk test, and parametric tests (ANOVA) followed by Tukey's post-hoc test were applied. The 15 donor pigs were considered as randomized complete blocks, and a pool of 3 wells was considered as an experimental unit. The statistical model of the porcine AM assays included LPS challenge, doses of seaweed extracts, and their interaction as fixed effects and included donor pigs (block) as a random effect. Pigs were used as a random factor, and cells collected from individual pigs were randomly used for experimental treatments (as a fixed effect). The data are expressed as least-squares means along with the standard error of the means. A probability value of < 0.05 was considered statistically significant. The graphs were created using GraphPad Prism 10 software (Boston, MA, USA).

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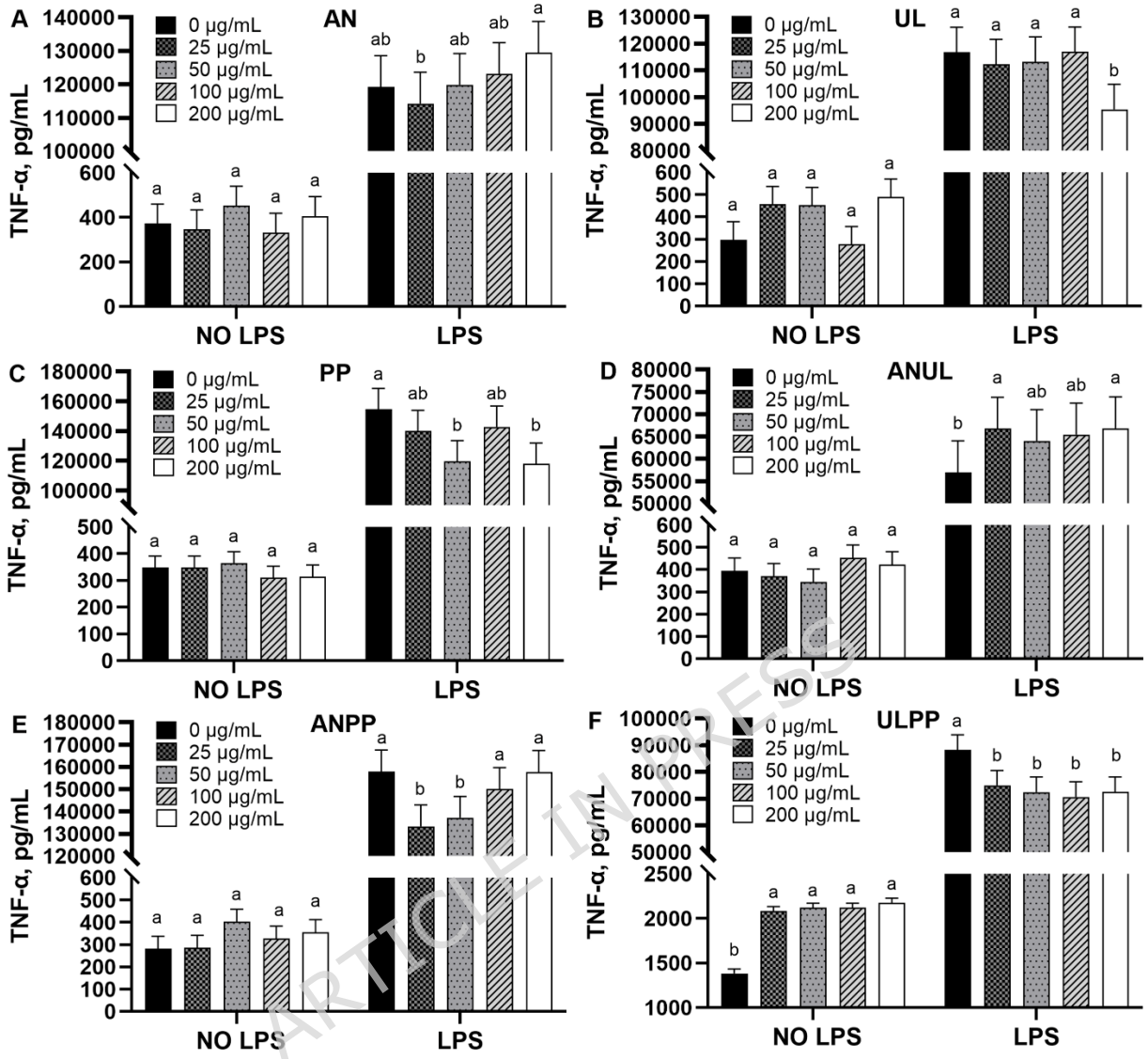

Fig. 1. Effect of *A. nodosum* (AN; A), *U. lactuca* (UL; B), *P. palmata* (PP;C), and their 1:1 combinations (ANUL; D), (ANPP; E), (ULPP; F) on the production of pro-inflammatory cytokine tumor necrosis factor-α (TNF-α; pg/mL) by porcine alveolar macrophages (porcine AMs) in the presence of lipopolysaccharide (LPS). Cells were incubated with different concentrations (0, 25, 50, 100, 200 µg/mL) of each seaweed extract, either with or without LPS (1 or 0 µg/mL) for 24 h. Different superscript letters indicate significant differences ($p < 0.05$) among treatments in the presence or absence of LPS. The results were means of values from 5 piglets.

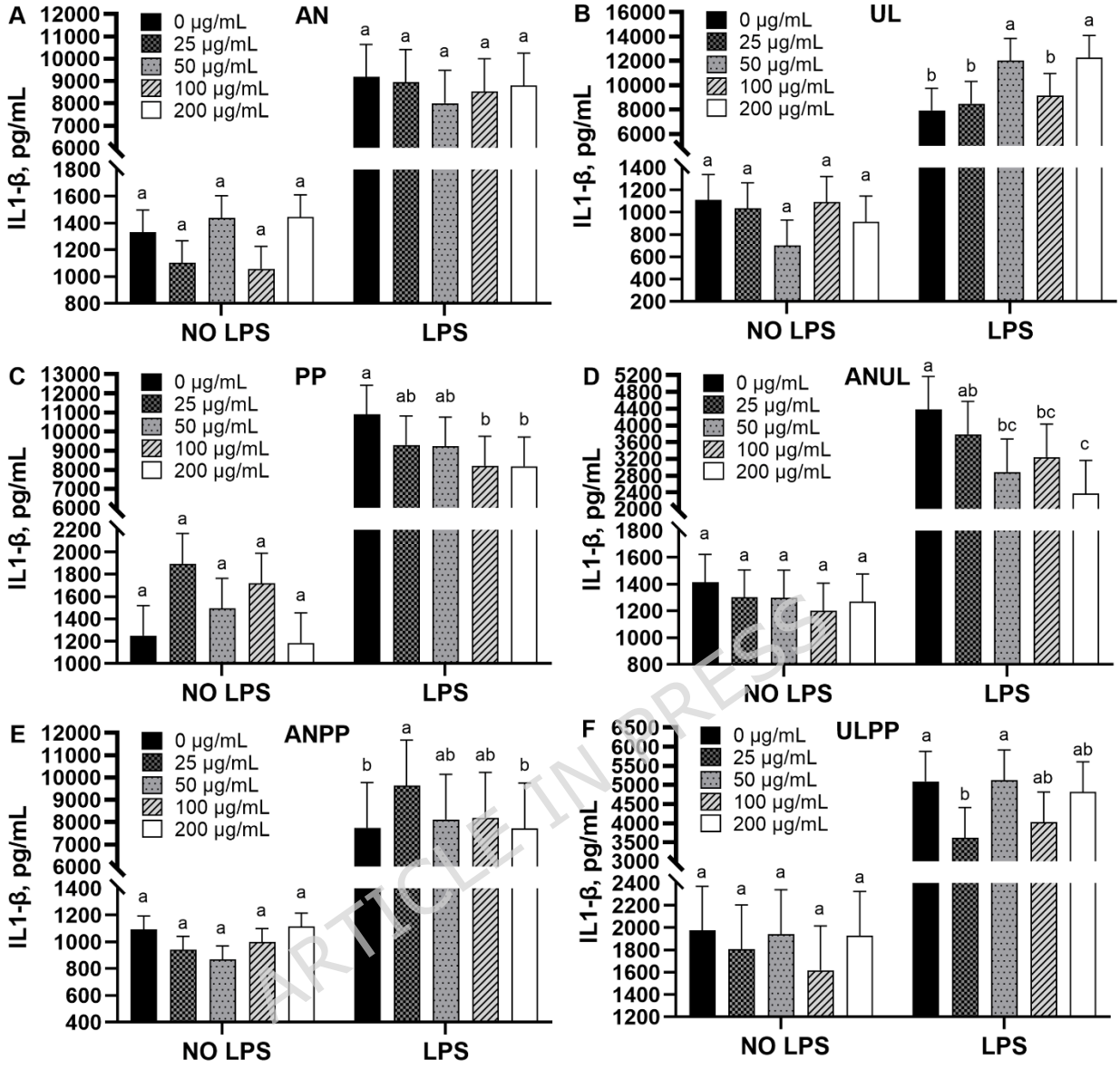

Fig. 2. Effect of *A. nodosum* (AN; A), *U. lactuca* (UL; B), *P. palmata* (PP; C), and their 1:1 combinations (ANUL; D), (ANPP; E), (ULPP; F) on the production of pro-inflammatory cytokine interleukin 1-β (IL-1β; pg/mL) by porcine alveolar macrophages (porcine AMs) in the presence of lipopolysaccharide (LPS). Cells were incubated with different concentrations (0, 25, 50, 100, 200 µg/mL) of each seaweed extract either with or without LPS (1 or 0 µg/mL) for 24 h. Different superscript letters indicate significant differences ($p < 0.05$) among treatments in the presence or absence of LPS. The results were means of values from 5 piglets.

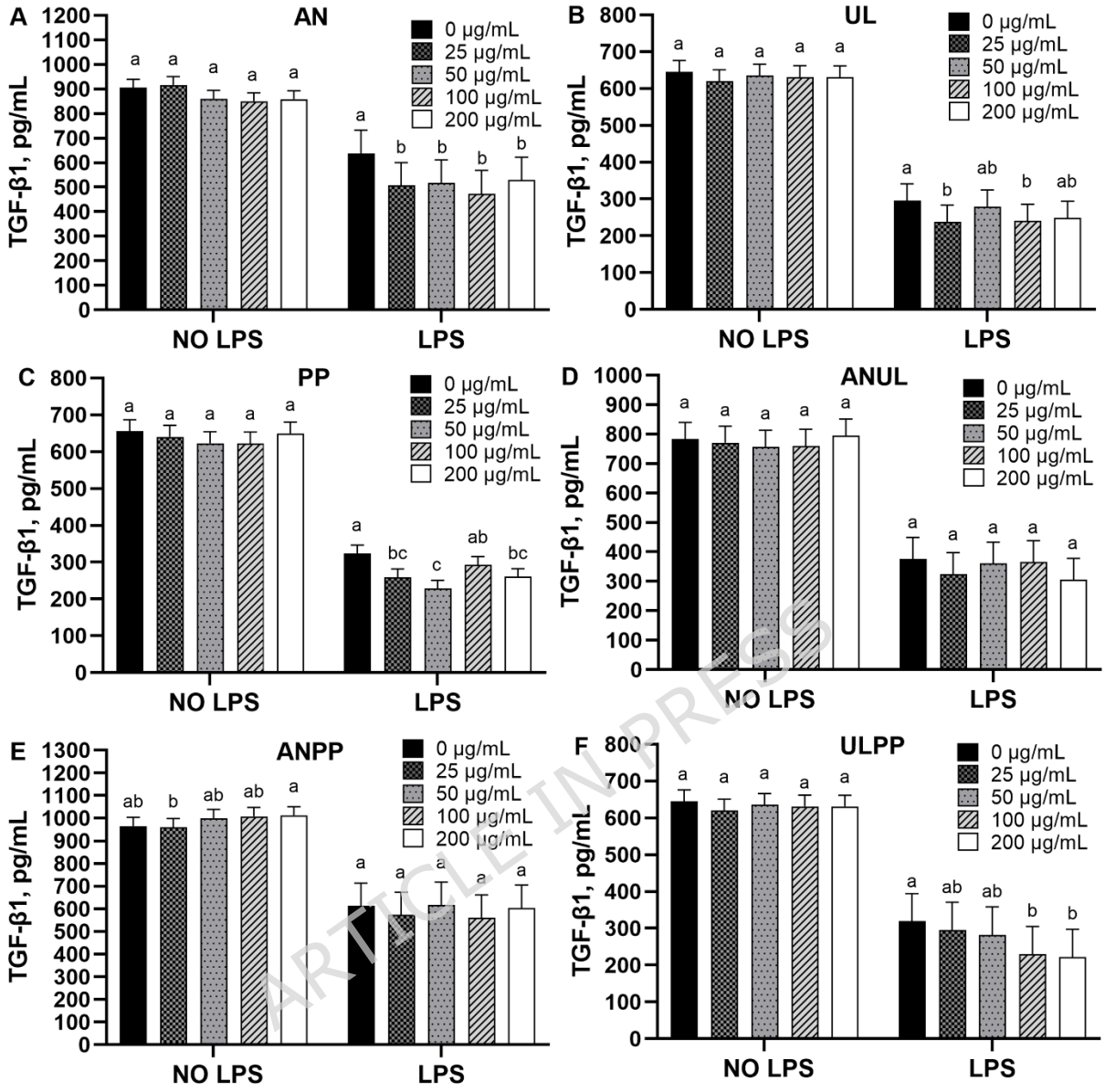

Fig. 3. Effect of *A. nodosum* (AN; A), *U. lactuca* (UL; B), *P. palmata* (PP; C), and their 1:1 combinations (ANUL; D), (ANPP; E), (ULPP; F) on the production of multifunctional cytokine transforming growth factor-β1 (TGF-β1; pg/mL) by porcine alveolar macrophages (porcine AMs) in the absence or presence of lipopolysaccharide (LPS). Cells were incubated with different concentrations (0, 25, 50, 100, 200 μg/mL) of each seaweed extract either with or without LPS (1 or 0 μg/mL) for 24 h. Different superscript letters indicate significant differences ($p < 0.05$) among treatments in the presence or absence of LPS. The results were means of values from 5 piglets.

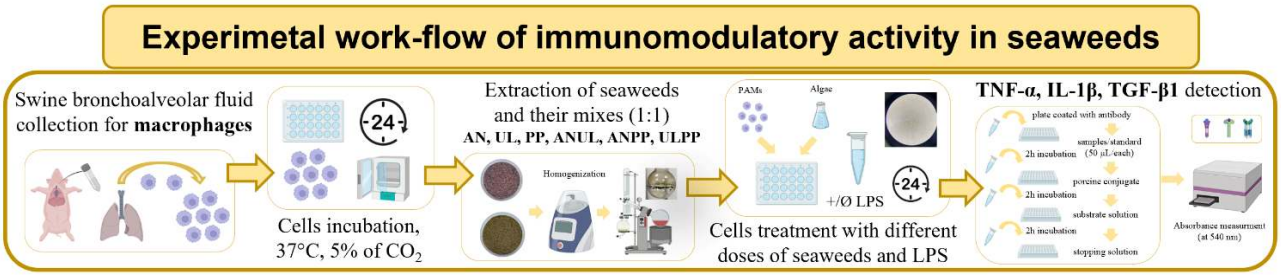

Fig. 4. The schematic presentation of the experimental work-flow (24 h indicating the 24 hour adaptation, and 24 h post-treatment). Created by M. Hejna, with selected graphical elements generated using BioRender.com (on May 2026).

Tab. 1. MTT cell viability (%) of porcine AMs with or without different seaweed extracts, with significant differences ($p < 0.05$) among treatments in the presence or absence of LPS. Data are presented as means ± standard error of the mean (SEM).

Seaweed extracts	Dose	Challenge		*p*-Values		
		NO LPS	LPS	Dose	Challenge	Interaction
PP	0	100.00 ± 0.000	98.37 ± 0.876	0.0040	0.9920	0.4642
	25	91.74 ± 7.924	104.68 ± 4.476			
	50	92.58 ± 3.357	106.23 ± 7.928			
	100	94.49 ± 3.488	105.15 ± 3.847			
	200	92.53 ± 5.148	102.78 ± 4.808			
UL	0	100.00 ± 0.000	101.61 ± 3.132	0.3256	0.7769	0.7709
	25	90.95 ± 12.057	95.67 ± 12.463			
	50	89.22 ± 6.531	104.81 ± 9.363			
	100	104.80 ± 5.919	100.06 ± 5.562			
	200	96.85 ± 6.440	103.39 ± 7.320			
AN	0	100.00 ± 0.000	98.36 ± 3.023	0.4587	0.8916	0.6479
	25	101.29 ± 12.719	87.01 ± 19.801			
	50	87.14 ± 16.488	90.06 ± 15.154			
	100	106.45 ± 8.839	91.70 ± 7.508			
	200	92.34 ± 11.970	92.40 ± 8.336			
ANPP	0	100.00 ± 0.000	99.36 ± 4.532	0.8623	0.9340	0.9908
	25	96.84 ± 11.279	100.89 ± 12.131			
	50	96.24 ± 12.094	93.47 ± 7.033			
	100	98.00 ± 12.349	95.16 ± 13.902			
	200	94.22 ± 13.732	100.28 ± 11.183			

ANUL	0	100.00 ± 0.000	99.99 ± 6.227	0.9943	0.9932	0.9526
	25	95.75 ± 22.256	99.32 ± 17.696			
	50	96.27 ± 13.988	100.70 ± 20.197			
	100	105.73 ± 16.622	94.04 ± 15.119			
	200	94.03 ± 17.997	97.99 ± 13.565			
ULPP	0	100.00 ± 6.628	97.14 ± 4.025	0.8759	0.6855	0.9600
	25	96.39 ± 10.276	103.75 ± 14.453			
	50	91.84 ± 11.707	89.02 ± 10.761			
	100	98.74 ± 6.148	90.79 ± 11.058			
	200	104.77 ± 8.874	103.27 ± 10.898			

7 Acknowledgments

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8 Author contribution

M. Hejna: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, roles/writing—original draft, writing—review and editing; **S. Frazzini**: investigation, formal analysis, writing—review and editing; **K. Wysocki**: investigation; **L. Rossi**: writing—review and editing; **J. Marchewka**: formal analysis; **M. Koszarska**: writing—review and editing**; A. Jóźwik**: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

9 Data Availability Statement

Data generated or analysed during this study are available from the corresponding author upon reasonable request.

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11 Competing Interests

The authors declare no competing interests.

12 Ethics approval

Not applicable.

13 Consent for publication

This study does not contain any individual personal data including any individual's name, images, or videos.

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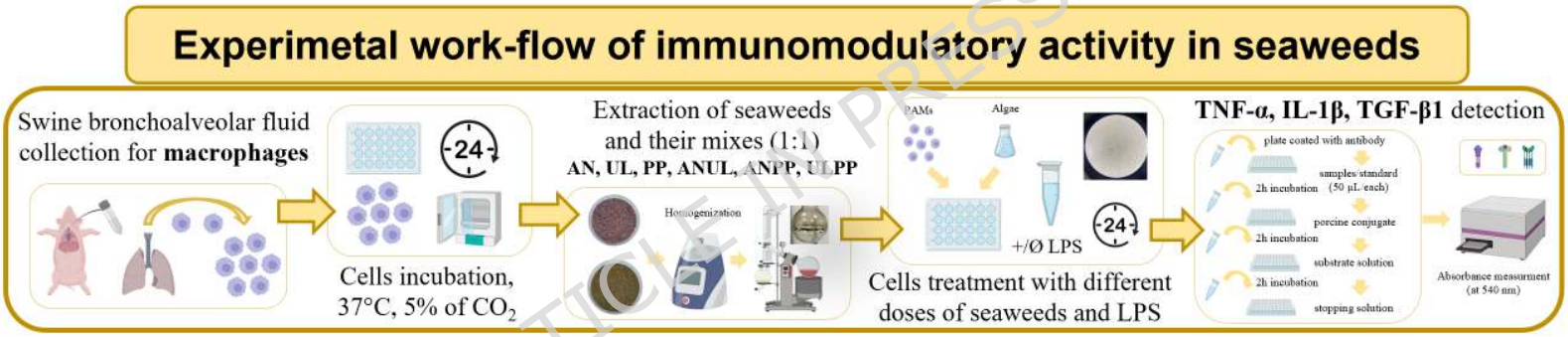

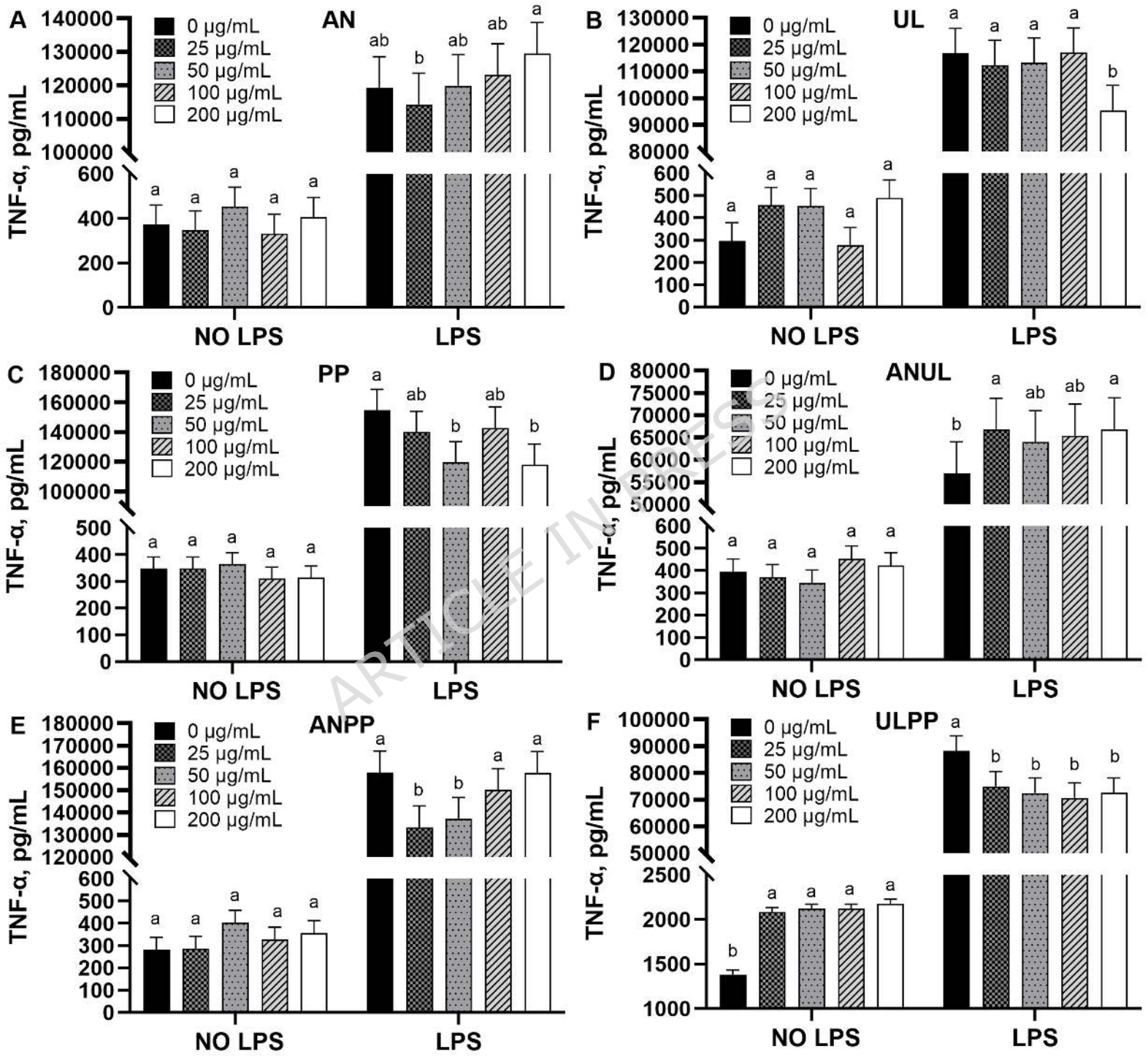

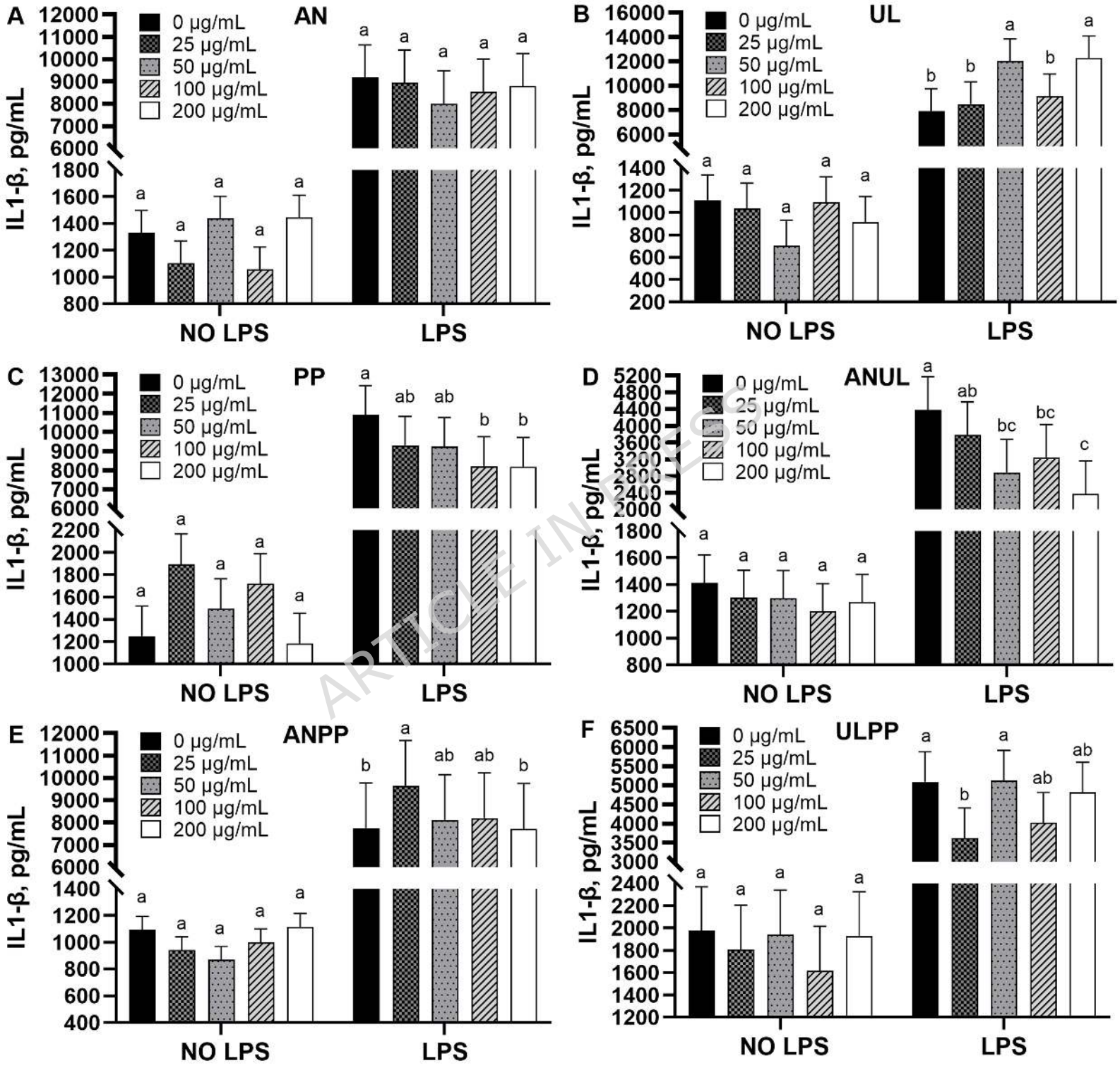

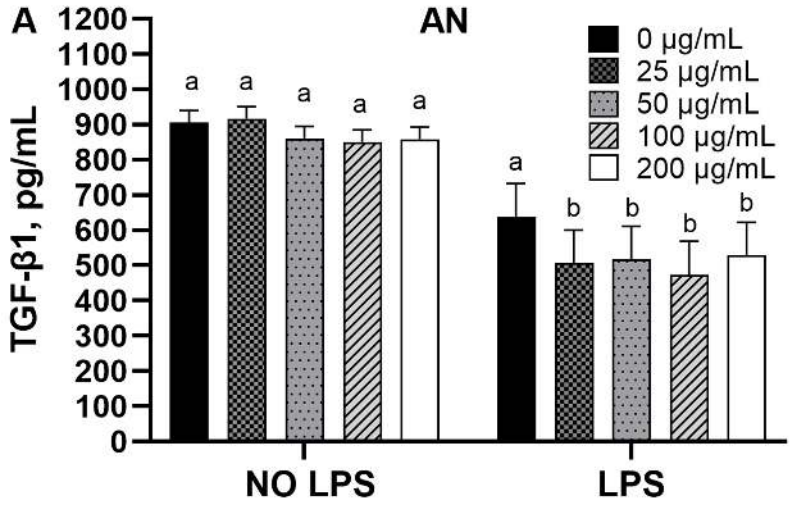

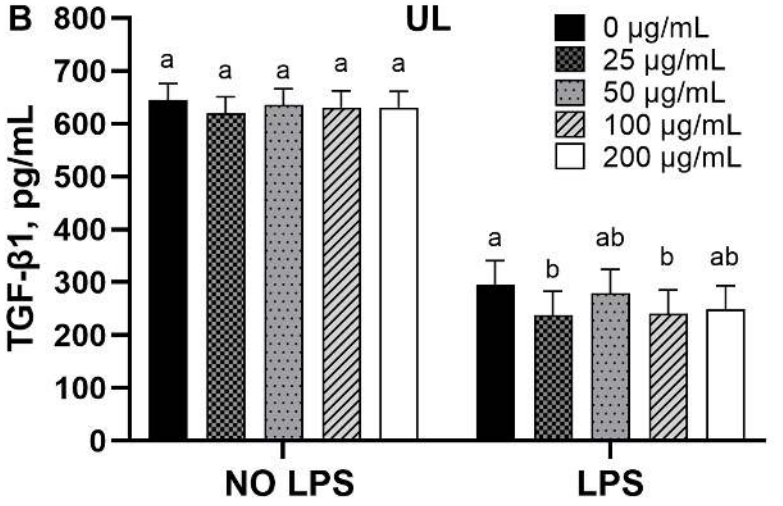

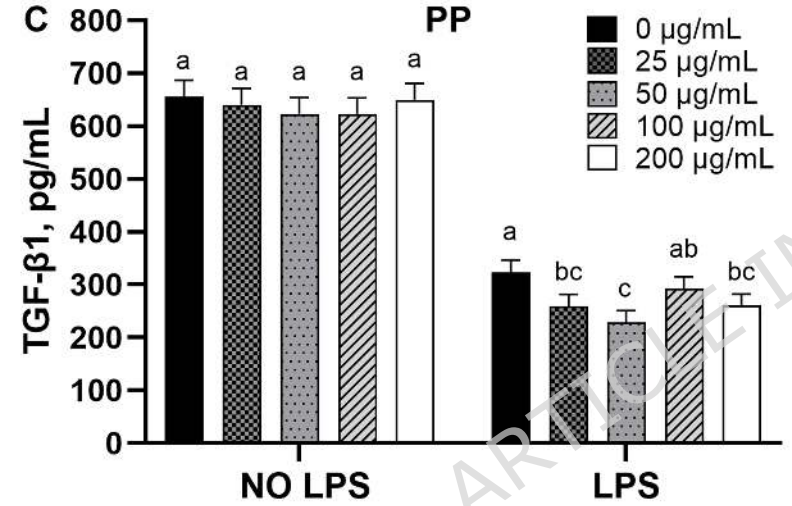

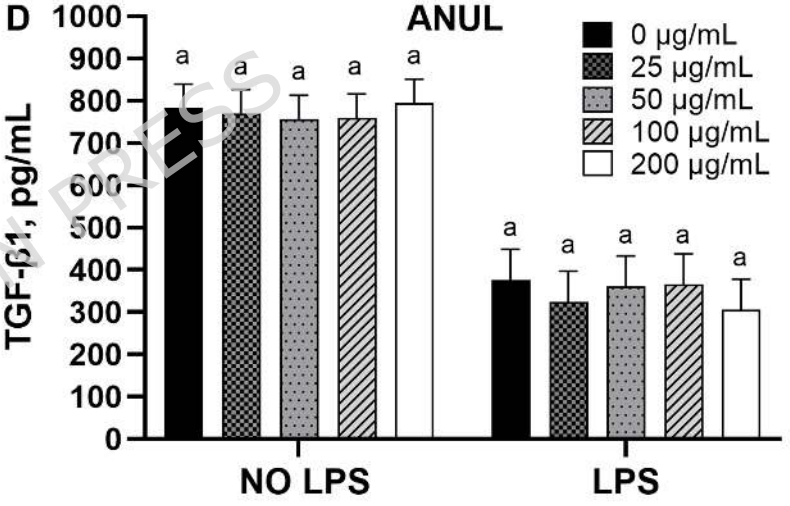

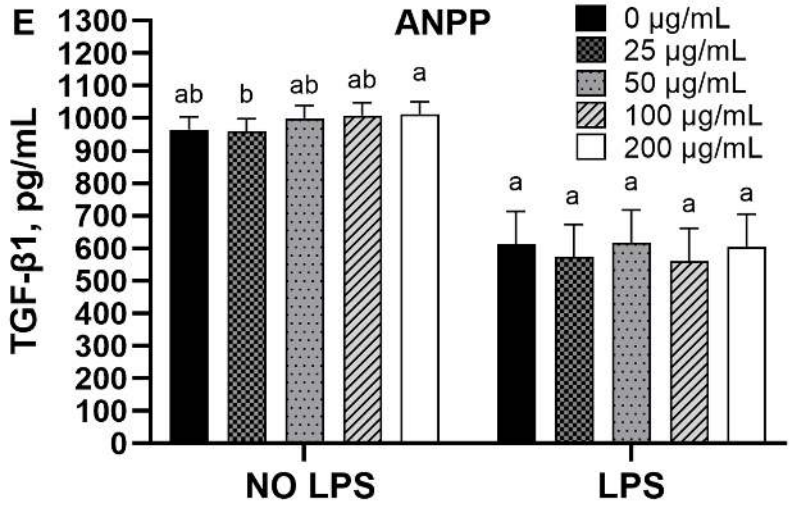

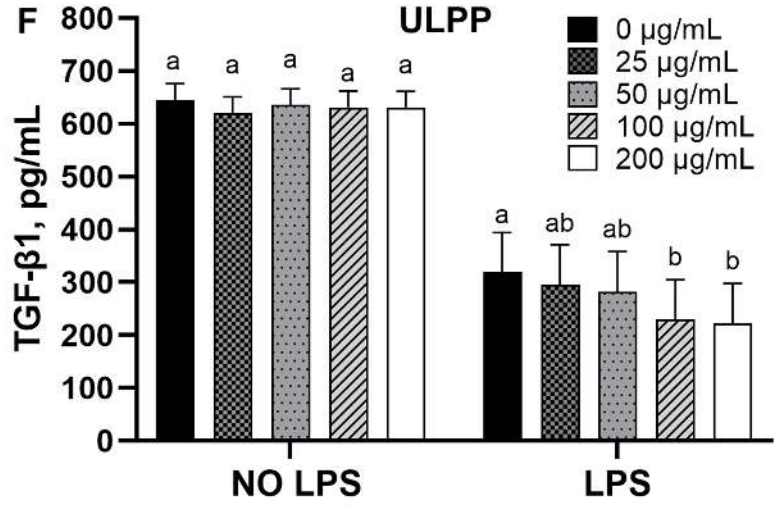

Tab. 1. MTT cell viability (%) of porcine AMs with or without different seaweed extracts, with significant differences ($p < 0.05$) among treatments in the presence or absence of LPS. Data are presented as means ± standard error of the mean (SEM).

Seaweed extracts	**Dose**	**Challenge**		***p*-Values**
		NO LPS	**LPS**	**Challenge**
PP	0	100.00 ± 0.000	98.37 ± 0.876	0.9920
	25	91.74 ± 7.924	104.68 ± 4.476	
	50	92.58 ± 3.357	106.23 ± 7.928	
	100	94.49 ± 3.488	105.15 ± 3.847	
	200	92.53 ± 5.148	102.78 ± 4.808	
UL	0	100.00 ± 0.000	101.61 ± 3.132	0.7769
	25	90.95 ± 12.057	95.67 ± 12.463	
	50	89.22 ± 6.531	104.81 ± 9.363	
	100	104.80 ± 5.919	100.06 ± 5.562	
	200	96.85 ± 6.440	103.39 ± 7.320	
AN	0	100.00 ± 0.000	98.36 ± 3.023	0.8916
	25	101.29 ± 12.719	87.01 ± 19.801	
	50	87.14 ± 16.488	90.06 ± 15.154	
	100	106.45 ± 8.839	91.70 ± 7.508	
	200	92.34 ± 11.970	92.40 ± 8.336	
ANPP	0	100.00 ± 0.000	99.36 ± 4.532	0.9340
	25	96.84 ± 11.279	100.89 ± 12.131	
	50	96.24 ± 12.094	93.47 ± 7.033	
	100	98.00 ± 12.349	95.16 ± 13.902	
	200	94.22 ± 13.732	100.28 ± 11.183	
ANUL	0	100.00 ± 0.000	99.99 ± 6.227	0.9932
	25	95.75 ± 22.256	99.32 ± 17.696	
	50	96.27 ± 13.988	100.70 ± 20.197	
	100	105.73 ± 16.622	94.04 ± 15.119	
	200	94.03 ± 17.997	97.99 ± 13.565	
ULPP	0	100.00 ± 6.628	97.14 ± 4.025	0.6855
	25	96.39 ± 10.276	103.75 ± 14.453	
	50	91.84 ± 11.707	89.02 ± 10.761	
	100	98.74 ± 6.148	90.79 ± 11.058	
	200	104.77 ± 8.874	103.27 ± 10.898	