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Functional characterization of antimicrobial and antioxidant properties of whole lemon pulp and depectinized lemon pulp as circular by-products in animal nutrition

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ABSTRACT

The increasing demand for sustainable livestock production underscores the need to explore innovative solutions that enhance resource efficiency and minimize waste. Agricultural by-products offer a valuable source of nutrients and bioactive compounds that can be effectively valorised for animal nutrition. This study investigated the nutritional composition, antioxidant, and antimicrobial properties of whole lemon pulp (WLP) and depectinized lemon pulp (DLP) as functional feed ingredients for animal health. WLP and DLP were characterized for bromatological composition through chemical analysis. Acetone (70 %, v/v), methanol (50 %, v/v), and water were used as extraction solvents before testing antioxidant capacity using the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolourisation assay and evaluating O138 *Escherichia coli* inhibitory activity through the microdilution method, monitoring bacterial growth spectrophotometrically. Obtained findings revealed that both WLP and DLP exhibited antioxidant activity, particularly for acetone and methanol extracts, showing markedly higher radical scavenging capacity than water extracts ($p < 0.0001$). Notably, WLP demonstrated increased antioxidant ability and inhibitory effects against verocytotoxic *E. coli* strain compared to DLP ($p < 0.0001$), likely due to the solubilization or denaturation of several functional compounds during the depectinization process in DLP. Despite this reduction, DLP retains residual antioxidant and antibacterial activity, potentially offering a cost-effective opportunity for utilization in animal nutrition within a circular economy strategy. These findings underscore the nutritional and functional potential of lemon by-products, suggesting further research into their applications in promoting animal health and sustainability in livestock production.

1. Introduction

World population growth, climate change, wars and pandemic-related implication drives the global food production through important challenges for the sector's sustainability [1]. It has been predicted that world population will reach 9.7 billion by 2050 [2]. Therefore, it is necessary to find innovative strategies to support the food demand, particularly for food from animal origin.

The concept of circular economy as a productive economic model with the purpose to reintroduce the waste to obtain sustainable by-products, can play a key role. One of the most important sectors for applying this model is the agro-industrial sector, where the reuse of waste biomass as by-products is becoming an important element for achieving a sustainable agriculture.

The increasing interest for the utilization of fruits and vegetables in animal nutrition is a result of this novel global view. Agricultural by-products promote circularity in the food-chain system and reduce the environmental impact of animal production [3]. Since conventional feedstuffs are often expensive, the utilization of agro-industrial by-products may be economically convenient for the farmers [4].

It is already known that several by-products have biologically active constituents, high nutritional and nutraceutical value, such as antioxidant compounds [5], anticancerogenic and antimicrobial molecules [6]. Several agricultural by-products can be used as feed ingredients to

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improve the quality of diets, animal health and performance, as well as the quality of milk and meat [7]. Among possible local Mediterranean by-products there is a wide availability of pomegranate, cocoa, carobs, hazelnut, pistachio, prickly pear, wine pomace and *citrus* by-product.

The genus *Citrus* spp. includes different types of fruits: orange, tangerine, lemon and grapefruit. *Citrus* fruits are principally consumed by humans as fresh fruit or processed juice. After fresh fruits are squeezed into juice, remain amounts to 50–70 % of the fresh weight of the original fruit and contains peel (60–65 %), internal tissues (30–35 %) and seeds (0–10 %) [8]. *Citrus* fruits are a natural source of pectin, which is commonly used in food industry as thickening, stabilizing, gel-forming or strength-building agent in the making of jam, fruit juice, ice cream and other products [9]. During the production of lemon juice, pectolytic enzymes are added to dissolve pectin enhancing the clarity of juice to meet consumer preferences [10]. Therefore, the resulting residue after industrial process is a lemon pulp with a depletion of pectin, known as depectinized lemon pulp. Whole lemon pulp (WLP) or depectinized lemon pulp (DLP), can be used in animal diets as a carbohydrate-rich ingredient with a high total digestible nutrient content, which averages around 74 %, a bulk energy feed with a high degree of water absorption, and it showed a good palatability for cattle [11]. Adding this by-product on ruminants' diet can represent an alternative source of energy in the concentrate feed [5]. As reported by Lashkari et al. [12], the chemical composition of different types of lemon pulp typically includes a crude protein content of 8.50 % on a dry matter basis, while the neutral detergent fiber (NDF) content for bitter lemon pulp was found to be 21.23 % on a dry matter basis. Similarly, Habeeb [13] reported an NDF content of approximately 20 % on a dry matter basis in lemon pulp, confirming the consistency of fibre levels across different sources. Ahmed et al. [14] suggested that *Citrus* pulp can be included up to 20 % in the concentrate of a total mixed ration (TMR) without any adverse impacts on rumen fermentation parameters. In addition, dried orange pulp can replace up to 75 % of yellow corn grains in Holstein dairy cows' diets without negative effects on nutrient digestibility, nutritive values and blood parameters [15].

The antioxidant properties of *Citrus* fruits are due to their ability to reduce the number of free radicals formed during oxidation processes [16]. Antioxidant activity is mostly dependent on the presence of phenolic compounds, such as phenolic acids, tannins, catechins and anthocyanins [17]. The marked antioxidant activity of some flavonoids seemed to be linked to the fact that the active chemicals are polyphenol compounds containing a chromanol ring system with the capacity to stabilize unpaired electrons and thereby scavenge free radicals [18].

Lemon (*Citrus limon*) is a widely cultivated citrus species, particularly prominent in the Mediterranean region and Turkey. Its pulp, a by-product of juice production in the food industry, is known to be a rich source of bioactive compounds, including phenolics, flavonoids, and coumarins. In the current study, lemon pulp was processed through drying and subsequent extraction using hot water and ethyl acetate. Lemon pulp has been considered as a promising source of limonoids and methoxylated flavones, known for their antioxidant properties. The antioxidant activity of both limonin and lemon pulp ethyl acetate extract was assessed through various *in vitro* assays, revealing strong dose-dependent antioxidant effects, including lipid peroxidation inhibition and free radical scavenging. These findings underscored the potential health benefits of lemon-derived bioactive compounds. According to Mathew et al. [19] *Citrus* lemon is a rich source of natural antioxidants, due to the redox properties present in polyphenols and flavonoids as another antioxidant family against free radical activity. In their *in vitro* antioxidant study shown that the peel fraction had stronger antioxidant efficacy, more reducing power ability and higher free radical scavenging activity with a better inhibition of the formation of lipid peroxides compared to pulp fractions. The study of Czech et al. [20] showed that the antioxidant activity of lemon determined using the $ABTS^{\bullet+}$ radical was significantly higher in the peel than in the pulp and for the content of phenolic acids respectively −0.273 and −0.358; for tannins 0.483 and −0.427; for ascorbic acid 0.519 and 0.013.

An important trial examined the antimicrobial activity of *Citrus* pulp by-product. Hot and cold aqueous in addition to ethanol extracts of *Citrus sinensis* (orange) peel were evaluated for their antimicrobial activity against some important pathogens from animals and poultry farms (*Staphylococcus aureus, Escherichia coli, Pseudomonas aerogenes, Bacillus cereus* and *Candida albicans*). Both hot, cold aqueous and ethanol peel extracts showed high antibacterial and antifungal effects against all pathogenic samples. *Citrus sinensis* (orange) peel extracts possess remarkable activity against gram-positive and gram-negative bacteria in addition to its antifungal activity against *Candida albicans* [21].

Recent study investigated the prebiotic potential of *Citrus* pulp by-product, as peels pectin content (20–30 %) is the main prebiotic oligosaccharide with several health benefits in the food industry [22]. Zafar et al. (2024) showed that prebiotic oligosaccharides extracted from citrus peel waste was an effective prebiotic as evidenced from its positive effect on growth of probiotic bacterial strains i.e. *Lactobacillus reuteri* and *Bifidobacterium*. Also, *Citrus* peel oligosaccharides suppressed the growth of pathogenic bacteria i.e., *E. coli*, during a co-culture with probiotic bacteria i.e., *Lactobacillus reuteri* and *Bifidobacterium*. Citrus peel prebiotic effect can be beneficial to promote *Lactobacillus reuteri* and *Bifidobacterium* and hindering the growth of *E. coli* [23].

However, despite various studies reporting positive outcomes in terms of production efficiency and health benefits from the supplementation of citrus by-products, the significant variability in their chemical composition has led to inconsistent results in both *in vitro* and *in vivo* trials. For this reason, it is essential to accurately characterize the properties of lemon residues to optimize their use in animal feed and maximize their beneficial effects on animal health. Therefore, this work aims to evaluate the functional characteristics of whole lemon pulp (WLP) and depectinized lemon pulp (DLP) with a particular focus on antibacterial and antioxidant capacity as sustainable by-products for animal nutrition.

2. Materials and methods

2.1. Processing of citrus pulp

Fresh lemon pulp as a by-product from Sicily, obtained after juicing different type of lemons, was prepared by Cargill Pectin Italy S.R.L. (Giammoro, Pace del Mela, Italy) in May 2024.

Lemon pulp, after juice and essential oil production, was received from Cargill Pectin Italy S.R.L. (Giammoro, Pace del Mela, Italy) for the pectin extraction. Upon arrival, the fresh peels were stored at room temperature before being grounded by 1 cm of sizing and washed with room temperature water. Subsequently, the peels underwent a squeezing process, and pectin extraction is started by adding nitric acid at final concentration 0,4–0,5 % (using HNO_3 65 %) and sodium carbonate. The solid-liquid separation results in the production of depectinized pulp. The production was verified and certified by SGS Italy S.P.A. (Milan, Italy) showing that the production compliances with the requisites of ISO 45001:2018 for the development and production of pectin for food and chemical use of by-products such as depectinized citrus for feed use.

The samples used in this study were collected both before and after the depectinization process to compare two different forms of the same product: fresh Whole Lemon Pulp (WLP) and fresh Depectinized Lemon Pulp (DLP). Both samples were obtained mixing four different subsamples to obtain a total of 8 kg for each WLP and DLP. Samples were shipped under refrigerated conditions and subsequently dried and homogenized before the proximate analysis.

2.2. Proximate chemical analysis

The analyses were carried out following the standard procedures from the Association of Official Analytical Chemists [24]. Lemon pulp

by-products, WLP and DLP were oven dried for 24 h at 65 °C until reaching constant weight for the evaluation of dry matter content (AOAC Official Method 934.01). Then dried samples were analyzed for crude protein (AOAC Official Method n. 2001.11), ether extract (AOAC Official Method n. 2003.05), neutral detergent fiber [25], crude fiber (AOCS method Ba 6a-05), and ash content (AOAC Official Method n. 942.05).

2.3. Extraction methods of WLP and DLP in different solvents

Prior to proceed with the characterization of functional activities, the bioactive molecules of citrus pulp and depectinized citrus were extracted using three different extraction solvents: deionized water, methanol (50 % diluted in deionized water, v/v), acetone (70 % diluted in deionized water, v/v). 2 g of samples meals were diluted in 10 mL of solvents (1:5 ratio v/v) and left stirred for 1 h at room temperature. The supernatants were collected after centrifugation (5000 rpm, 10 min), filtered through a 0.45 μm syringe filter and stored at −20 °C until the analysis. Extraction procedures were performed in duplicate.

2.4. Trolox Equivalent Antioxidant Capacity assay (TEAC) and polyphenols content

A Trolox Equivalent Antioxidant Capacity (TEAC) assay was performed to evaluate the antioxidant effect of extracted samples. For the TEAC analysis, a 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma-Aldrich, St. Louis, MO, USA) radical cation decolourisation test was performed. $ABTS^{\bullet+}$ was generated by diluting potassium persulfate in ABTS (7 mM) to obtain a final concentration of 2.45 mM. The reaction was left to stand in the dark for 16 h at room temperature. $ABTS^{\bullet+}$ was diluted in MilliQ water to obtain the working solution with an optical density (OD) of 0.70 ± 0.02 at 734 nm at room temperature. A concentration series (from 2000 to 125 μM) of Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid; supplied by Sigma-Aldrich, St. Louis, MO, USA) was used to build the calibration curve. Samples and standards were tested by mixing 10 μL with 1000 μL of diluted $ABTS^{\bullet+}$ working solution in a test tube. Absorbances were recorded using a UV–Vis spectrometer at 734 nm after 6 min of reaction (Jasco V-630 UV–Vis/NIR Spectrophotometer, Jasco Inc. LTD., Tokyo, Japan). The Trolox calibration curve was used to express data as μmol of Trolox equivalents on a mg of citrus pulp extracts (μmol TroloxEq/g). All determinations were performed in triplicate.

2.5. Polyphenols content assay

The total polyphenols content was determined using a microtiter plate assay according to Attard [26]. Briefly, the extracts were tested for their phenolic content based on their reaction with the Folin-Ciocalteau reagent in presence of alkaline environment provided by sodium carbonate addition. Specifically, the reaction mixture consisted of 10 μL of the sample, 100 μL of Folin-Ciocalteau reagent (previously diluted 1:10, v/v in deionized water), and 80 μL of Na_2CO_3 (1 M). Absorbances were read at 630 nm after 20 min of incubation at room temperature with a microtiter plate reader (BioTek Epoch Microplate Spectrophotometer, Agilent Technologies, Santa Clara, CA, USA). A standard curve, prepared using different concentrations of tannic acid concentrations (960, 480, 240, 120, 60, 30, 15, 7.5 and 0 μg/mL), was used to calculate the results expressing data as mg of tannic acid equivalents/100 g of sample (mg TA Eq/100g). All determinations were performed in triplicate.

2.5.1. Total flavonoid content assay (TFC)

TFC of WLP and DLP extracts was evaluated according to Herald using the aluminium chloride for obtaining a colorimetric reaction [27]. The calibration curve was prepared in 1:2 serial dilutions, from 250 μg/mL to 7.81 μg/mL, using catechin as the standard and optical densities were recorded at 510 nm (BioTek Epoch Microplate Spectrophotometer, Agilent Technologies, Santa Clara, CA, USA). TFC was expressed as mg catechin equivalent (CEq)/g of sample (mg CEq/g). Each sample and standard were run in triplicate.

2.6. Growth inhibitory assay against verocytotoxic Escherichia coli

A liquid culture-based of F18 + *Escherichia coli* (*E. coli*) growth inhibition assay was performed to estimate the inhibitory activity of previously extracted citrus samples biomass at different concentrations. A verocytotoxic *E. coli* strain, expressing F18 adhesive fimbria, was used from the strain collection of the Department of Veterinary Medicine and Animal Sciences of University of Milan. Verocytotoxic *E. coli* strain was used as reference model for pathogenic bacteria expressing virulence factors (verocytotoxin type 2 and F18 adhesive fimbria) responsible of severe clinical symptoms. Bacteria were cultured overnight at 37 °C under stirring (150 rpm) in Luria-Bertani broth (LB) medium under an aerobic condition, serving as the inoculum for all subsequent experiments. The overnight *E. coli* culture was used as inoculum in 96 microplates wells of containing 100 μL of LB medium supplemented with different doses of extracts (25 % v/v, 12.5 % v/v, 6.25 % v/v) diluted in LB medium of lemon pulp and depectinized lemon pulp. Before inoculation, bacterial cultures were standardized to initial density (0.05 ± 0.02 OD when read against LB medium) using a spectrophotometer at λ = 600 nm. Microplates were incubated aerobically at 37 °C for 6 h. The bacterial growth was measured via measurement of the optical density of each culture at 620 nm (OD620) at 60 min intervals in a spectrophotometer (ScanReady P-800, Life Real, Zhejiang, China). Control wells including the same concentration of extraction solvents inoculated with bacteria were used to provide a proper comparison of *E. coli* growth in absence of bioactive compounds. Bacteria-free wells with equivalent concentrations of citrus extracts were used as blanks to subtract the background turbidity caused by extract colour. All data acquired from the optical density measurement were converted to log-transformed colony forming units per mL (CFU/mL) using a calibration curve obtained by plate counting (data not shown) each hour to convert the OD value into number of CFU/mL. The assay was performed in four technical replicates. The increase in absorbance determined bacterial growth.

2.7. Growth of Lactiplantibacillus plantarum and Limosilactobacillus reuteri supplemented with whole lemon pulp and depectinized lemon pulp aqueous extract

The effect of WLP and DLP was evaluated on the growth of *Lactiplantibacillus plantarum* and *Limosilactobacillus reuteri* according to Frazzini et al. [28]. Briefly, lactic, *L. plantarum* and *L. reuteri* strains were incubated for 6 h lactic acid strains starting from a concentration of 10^7 CFU/mL, in MRS broth (Merck, Germany), in the presence of WLP and DLP extracts at 25 % concentration. The growth of *L. plantarum* and *L. reuteri* was determined via measurement of the optical density of each culture at 600 nm (OD600) at specific intervals during incubation at 35 °C in a spectrophotometer (P-800 Scan Ready, Life Real). All obtained data were converted into $\log_{10}$ of the number of CFU/mL, by a calibration curve (considering 1 OD equal to 10^9 CFU/mL) [29].

2.8. Statistical analysis

The results were analyzed using GraphPad Prism software, version 9.0. Data were preliminarily evaluated for normal distribution with the Kolmogorov–Smirnov test. Due to the low number of replicates (n = 2 per sample type measured for three or four technical determinations), antioxidant activity, polyphenol and flavonoids content were assessed using the Kruskal-Wallis test to examine differences among the various extracts of LPW and LPSP samples, with medians subsequently compared using Dunn's multiple comparisons test. The parametric approach, involving ANOVA followed by Tukey's Honestly Significant

Difference (HSD) test, was then adopted as it produced the same output as the non-parametric test for the antioxidant activity dataset. This approach was retained to ensure higher power of test with lower number of data and controlling the probability of committing a Type II error, and the homogeneity of variances was verified using Bartlett's test.

Bacterial growth data from the growth inhibitory test of *E. coli* and probiotic activity with *L. plantarum* and *L. reuteri* were analyzed using a mixed model that included the fixed effects of time, treatment, and their interaction (treatment × time). Means were compared using Sidak's post-hoc test to highlight differences among treatments for each time point.

Data are presented as means ± standard error, with means considered significantly different at $p \leq 0.05$.

3. Results and discussion

In this study, the chemical composition and the antimicrobial and antioxidant properties of whole lemon pulp and depectinized lemon pulp were investigated as agro-industrial by-products to evaluate their specific characteristics for further use as potential feed ingredients with a beneficial impact on animal health.

3.1. Nutritional characteristics of whole lemon pulp and depectinized lemon pulp

Table 1 shows that both samples of WLP and DLP were characterized by a low content of moisture due to the oven dried process made before chemical analysis. DLP revealed increased content of crude fiber (CF) and NDF than WLP after the depectinization procedure. The content of crude protein (CP) is comparable for both sample and could be relevant in the perspective to use them in animal nutrition. Ether extract showed higher value in DLP compared to WLP, in contrast to the ashes value which exhibit higher content for WLP than DLP.

WLP and DLP were primarily characterized by their CP content, which was approximately 8 %, and their high levels of NDF which registered values of 27.44 % and 61.53 % on a dry matter basis, respectively. According to Mhgub et al. [30] DLP showed a content of NDF higher than WLP due to the process of depectinization. On the other hand, Pham et al. [31] registered a smaller quantity of crude protein (3.59 ± 0.19 %) in WLP compared to our data. In contrast to Janati et al. [32] lemon peel showed a lower percentage of NDF and the ether extract exhibited higher value and comparable ash content compared to WLP investigated in this study. DLP and WLP as a circular by-product, can be used in animal feeding due to their important nutritional value. DLP is a rich source of NDF, an important fiber useful for ruminant digestibility. According to Benestante et al. [33] fresh lemon by-product have a huge quantity of moisture that after drying process is mostly lost. Regarding nutritional characteristics, our study aligns with previous research analysing both whole and depectinized lemon pulp. The substantial NDF content in DLP could be particularly valuable for ruminants, highlighting the need for thorough nutritional profiling of WLP and DLP, given their significant variability in the market. Ether extract and ashes contents, representing the lipid component and inorganic matter, were the lowest among the measured parameters (both <7 %). While these values are relatively low, their contribution to the overall nutritional value of the LPW and DLP is likely limited if supplemented in a complete feed formulation.

Table 1
Chemical analysis of lemon pulp dried samples.

	WLP	DLP
Moisture %	4.52	3.96
Crude Protein %	8.41	8.70
Ether Extract %	1.67	4.77
Neutral Detergent Fiber %	27.44	61.53
Crude Fiber %	15.01	39.82
Ashes %	5.52	2.01

Data are reported as means of duplicate determinations on dry matter basis. WLP: Whole lemon pulp, DLP: Depectinized lemon pulp.

The different chemical compositions of lemon fruits can be attributed to variability in regional characteristics and harvest seasons, which ultimately affect the final product profile. Therefore, it is essential to characterize the available by-products for their potential use in animal nutrition to ensure a proper nutritional balance and functional compounds supplementation [34].

3.2. Antioxidant activity and polyphenols content of whole lemon pulp and depectinized lemon pulp extracts

WLP and DLP showed a higher antioxidant activity considering the 70 % acetone and 50 % methanol extracts compared to the simple aqueous extract ($p < 0.0001$). WLP revealed significantly higher radical scavenging activity compared to DLP for each extraction solvent tested ($p < 0.0001$). Specifically, WLP showed a value of 52.30 ± 2.32 μTrolox Eq/g for acetone extraction, 52.18 ± 1.76 μTrolox Eq/g for methanol and 35.92 ± 0.79 μTrolox Eq/g for water extraction. On the other hand, DLP revealed a content of 4.35 ± 0.23 μTrolox Eq/g for acetone extraction, 4.15 ± 0.06 μTrolox Eq/g for methanol extraction and 1.51 ± 0.12 μTrolox Eq/g for water extraction.

In line with the antioxidant activity, the phenolic content in WLP showed higher concentrations of polyphenols in acetone 70 % and methanol 50 % extracts compared to the water used as solvent ($p < 0.0001$; Fig. 1b). DLP revealed the highest content of polyphenols in acetone 70 % extracts compared to methanol and water solvents ($p < 0.0001$). Contemporary, water extract showed the lowest concentration in phenolic compounds compared to methanol 50 % and acetone 70 % extracts ($p < 0.0001$). Accordingly, the flavonoids content was in line with phenolic content showing the same significances among different extracts indicating a direct relationship among the content of polyphenols and flavonoids in the WLP and DLP samples ($p < 0.0001$; Fig. 1cc). Water showed limited efficiency in extracting flavonoids, likely due to its lower ionic strength and reduced ability to solubilize less polar or moderately polar compounds if compared to acetone and methanol.

Antioxidant capacity may be an important attribute when assessing the potential benefits of by-products in animal feeding [35]. In this study, acetone and methanol extracts demonstrated higher antioxidant activity compared to water-based extracts, suggesting the presence of both polar and nonpolar antioxidant compounds in WLP and DLP contributing to radical scavenging ability. The lower antioxidant capacity observed in water extracts may be due to the limited potential to mainly solubilize polar molecules. In contrast, methanol and acetone can extract a broader range of compounds, including those less soluble in a purely aqueous environment, thereby increasing the yield of antioxidant compounds that contribute to the overall scavenging activity [36]. Similar findings have been reported in other studies, where methanol and acetone extracts of natural products, such as pomegranate peels and date seeds, showed higher antioxidant activities compared to aqueous extracts [37,38]. Notably, DLP exhibited a substantial reduction in radical scavenging capacity, with over 90 % lower activity compared to the equivalent WLP extract. This decline is likely due to the depectinization process, which solubilizes pectin and others hydrosoluble compounds. Additionally, the treatment of nitric acid can play a role denaturing residual polyphenols and other antioxidants in DLP [39]. Our findings indicate that depectinization process likely denatures or solubilizes several antioxidant compounds in WLP, leading to lower single electron transfer activity in DLP [40]. The polyphenols concentration confirmed the results obtained for radical scavenging activity. Polyphenols, characterized by hydroxyl groups bounded to aromatic rings, can donate hydrogen atoms or electrons to neutralize free radicals, thereby interrupting oxidative chain reactions [41]. This suggests that the primary scavenging activity in the extracts is likely attributable to

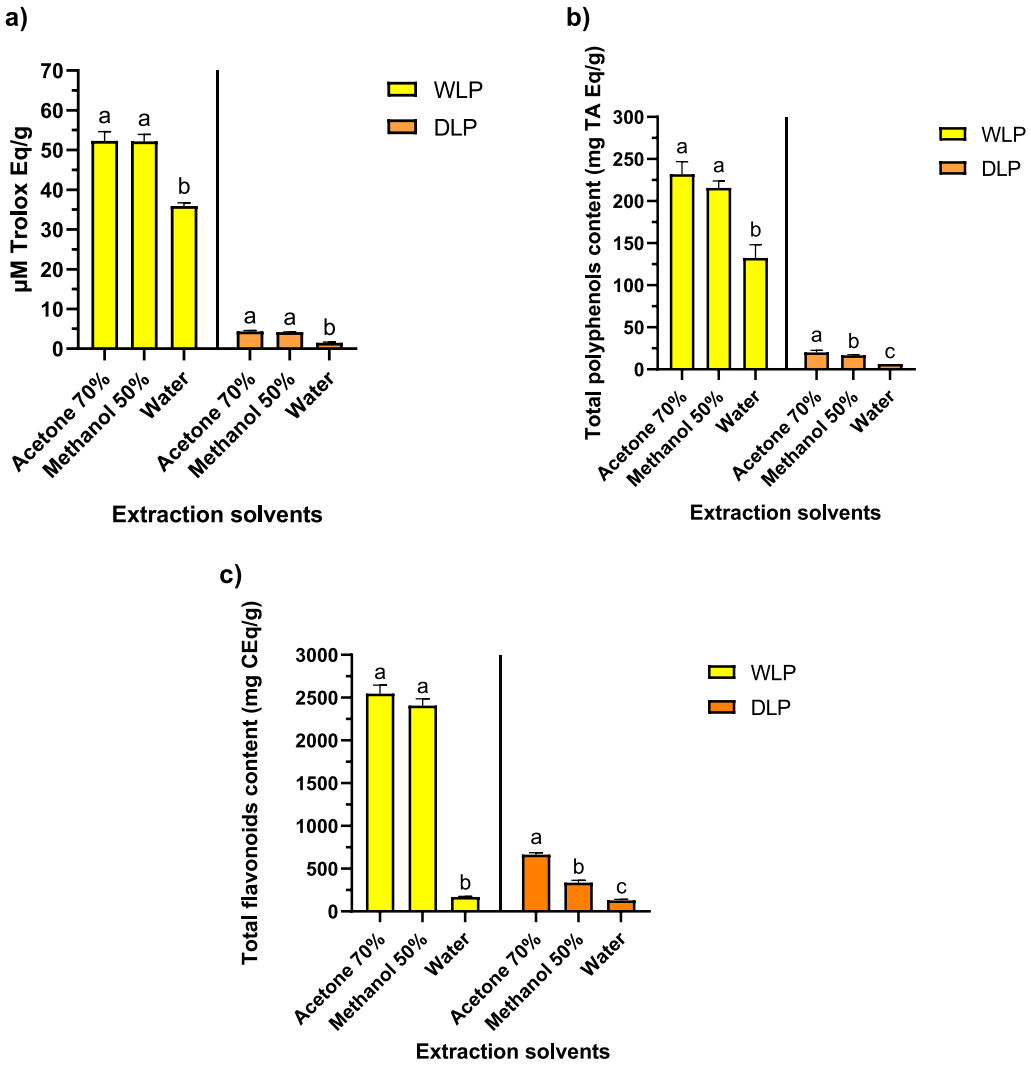

Fig. 1. Antioxidant activity, polyphenols and flavonoids content of whole lemon pulp (WLP) and depectinized lemon pulp (DLP) measured in three different extracts (acetone 70 %, methanol 50 %, and water). **a)** Trolox equivalent antioxidant capacity of WLP and DLP extracts. **b)** Total polyphenols content of WLP and DLP extracts. **c)** Total flavonoids content of WLP and DLP extracts.
Data are presented as means ± standard deviation. [a-b-c] Means with different lowercase letters indicate statistically significant differences among tested groups ($p < 0.0001$).
TA Eq: tannic acid equivalents; CEq: catechin equivalents.

the phenolic moieties. This relationship has been widely described in different plant-based extracts, where higher phenolic content directly correlated with increased radical scavenging efficiency even though not all polyphenols contribute to the antioxidant capacity. It is noteworthy that the effectiveness of polyphenols can also depend on their structural features, including the degree of hydroxylation, presence of methoxy groups, and conjugation with other bioactive molecules, which influence their redox potential [42]. Further studies aimed at identifying the specific classes of polyphenols involved, will be useful for fully elucidating the potential benefits of lemon by-products.

3.3. Growth inhibitory activity against verocytotoxic E. Coli of WLP and DLP extracts

WLP and DLP extracts exerted different antimicrobial activities depending on the extraction solvents (Fig. 2). Particularly, acetone extract showed inhibitory activity of WLP starting at 3 h of incubation until 6 h of reaching inhibitory values of about 99 % if compared to the control testing all the three concentrations. DLP showed lower inhibitory activity when compared to WLP after 5 and 6 h when added to the medium at 12.5 %, reaching an inhibition of approximately 60 % if compared to the control after 6 h ($p < 0.0001$; Fig. 2b). Additionally, at 6.25 % acetone extract of DLP showed decrease inhibitory activity from 3 to 6 h even if this compound was still able to extend the time for the binary fission of *E. coli* when compared to the control revealing an inhibition lower than 20 % at the sixth hour ($p < 0.0001$; Fig. 2c). Methanol extract showed no bacterial growth when the extracts and methanol were added to the medium at 25 % without highlighting any bacterial growth for 6 h (Fig. 3a). The 12.5 % methanol extract of WLP showed lower bacterial growth from 3 to 6 h if compared to both 12.5 % DLP and *E. coli* ($p < 0.0001$; Fig. 3b and Fig. 3c). Both 12.5 % and 6.25 % DLP methanol extract showed intermediate effects if compared to the same concentrations of WLP and *E. coli* from 3 to 6 h for incubation reaching values of inhibition of 50 % and 18 % at 6 h, respectively ($p < 0.0001$). Water extraction of WLP showed significant inhibition against *E. coli* growth from 2 to 6 h of testing for all concentrations tested ($p < 0.0001$; Fig. 4). However, it is important to underline that water does not possess intrinsic antibacterial activity, and the control groups incubated in LB supplemented with water used as control showed the highest bacterial growth. This makes the inhibitory effect of WLP water extract

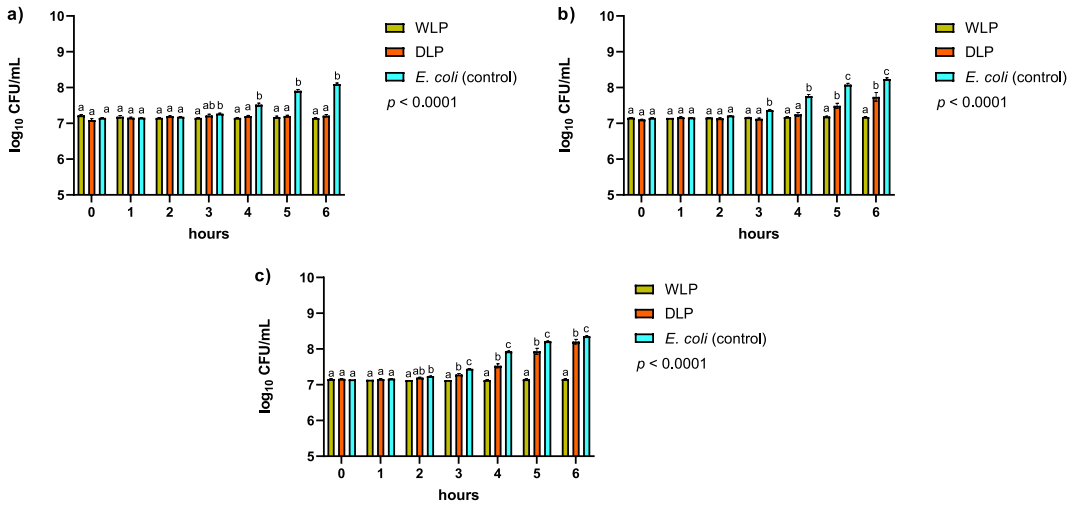

Fig. 2. *Escherichia coli* growth inhibitory activity of acetone (70 %, v/v) extracts of whole lemon pulp (WLP) and depectinized lemon pulp (DLP). **a)** 25 % of acetone extracts of WLP, DLP and 25 % of acetone for *E. coli* control. **b)** 12.5 % of acetone extracts of WLP, DLP and 12.5 % of acetone for *E. coli* control. **c)** 6.25 % of acetone extracts of WLP, DLP and 12.5 % of acetone for *E. coli* control.

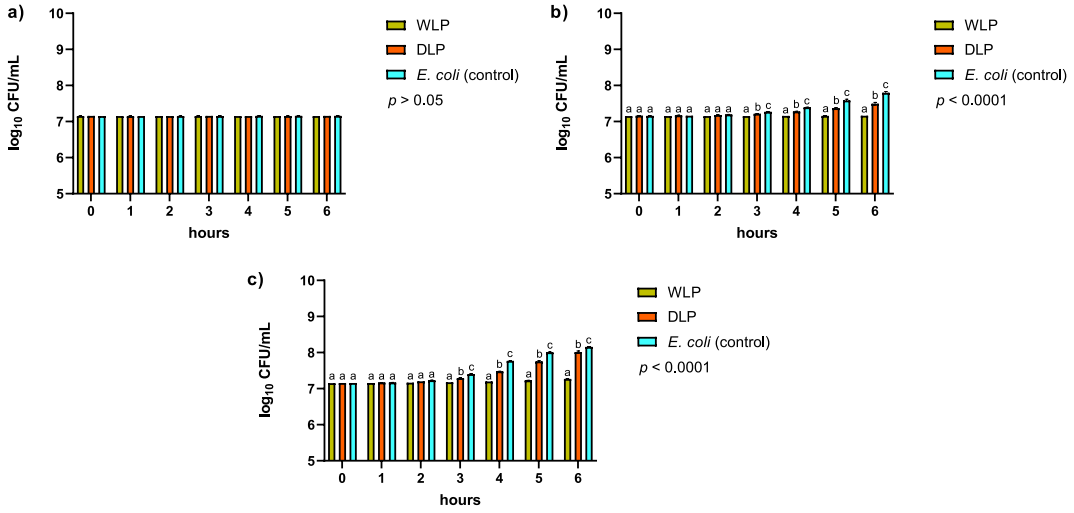

Fig. 3. *Escherichia coli* growth inhibitory activity of methanol (50 %, v/v) extracts of whole lemon pulp (WLP) and depectinized lemon pulp (DLP). **a)** 25 % of methanol extracts of WLP, DLP and 25 % of methanol for *E. coli* control. **b)** 12.5 % of methanol extracts of WLP, DLP and 12.5 % of methanol for *E. coli* control. **c)** 6.25 % of methanol extracts of WLP, DLP and 12.5 % of methanol for *E. coli* control.

interesting, as it reflects the presence of free active polar compounds. Regarding DLP extracts 25 % of water extract showed a lower inhibitory activity if compared to 25 % of WLP water extract from 3 to 6 h slowly decreasing the inhibitory effect over time reaching an average inhibition of 26 % at 6 h if compared to the control ($p < 0.0001$, Fig. 4a). On the opposite hand, 12.5 % of DLP water extracts revealed comparable bacterial growth if compared to *E. coli* from 0 to 4 h of incubation and after 5 h 12.5 % of DLP water extract showed increased bacterial growth if compared to control *E. coli* ($p < 0.0001$, Fig. 4b). Similarly, 6.25 % of DLP water extract displayed a comparable bacterial growth compared to *E. coli* from 0 to 3 h of incubation and a significant raised growth from 4 to 6 h of test if compared to the control ($p < 0.0001$, Fig. 4c).

The evaluation of antibacterial activity is critical for assessing the functional properties of by-products, as one of the most effective strategies for reducing antibiotic use is through the dietary supplementation of compounds able to inhibit pathogens. This approach significantly reduces the incidence of various multifactorial, gastrointestinal and systemic diseases [43]. Verocytoxin strains of *E. coli* express important virulence factors, leading to several clinical signs, high morbidity and mortality rates in both animals and humans [44]. The specific strain O138 *E. coli* plays a key role in the post-weaning disease and oedema disease in pigs, represents a major risk factor in swine farming which drives veterinarians to rely on antibiotics to limit the detrimental effects of this pathogen on piglets [45,46]. Furthermore, this bacterial strain can be considered as useful model for translational studies on similar verocytotoxigenic strains, such as zoonotic O157:H7 *E. coli* strain, which produces similar toxins cause of severe foodborne diseases in humans [47], leading to symptoms such as bloody diarrhoea and haemolytic uremic syndrome in paediatric patients, which can be life-threatening [48].

Our findings indicated that the acetone extract of WLP exhibited the strongest inhibitory activity against *E. coli*, with a complete growth suppression observed for all tested concentrations up to 6 h. In contrast, the 12.5 % acetone extract of DLP showed a gradual loss of activity over time, although it still revealed a lower bacterial growth compared to the control. Methanol extracts of both WLP and DLP effectively inhibited bacterial growth. However, the reduction in microbial growth was consistently higher in WLP compared to DLP also with methanol extract. Specifically, the methanol extract at 6.25 % concentration completely inhibited *E. coli* growth in the WLP-supplemented medium, while the

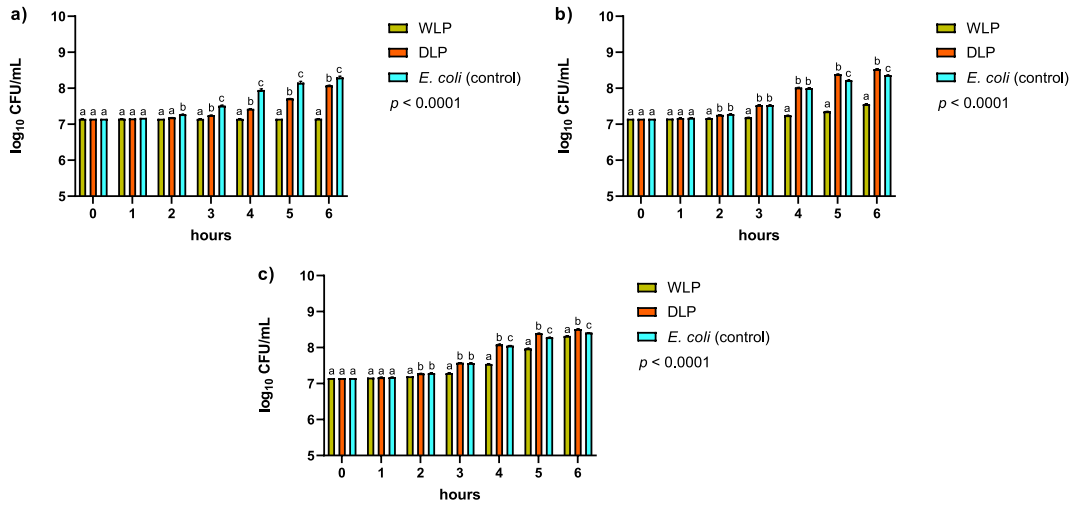

Fig. 4. *Escherichia coli* growth inhibitory activity of water extracts of whole lemon pulp (WLP) and depectinized lemon pulp (DLP). **a)** 25 % of water extracts of WLP, DLP and 25 % of water for *E. coli* control. **b)** 12.5 % of water extracts of WLP, DLP and 12.5 % of water for *E. coli* control. **c)** 6.25 % of water extracts of WLP, DLP and 12.5 % of water for *E. coli* control.

DLP supplemented medium showed bacterial growth but consistently lower than the control for 6 h. The consistent inhibitory effect observed with methanol and acetone extracts discloses that organic solvents enhance the extraction of bioactive phytochemicals responsible for inhibitory activity, unlike water which may be limited in solubilizing a wide array of different compounds. In line with our study Dhanavede et al. [48] found that the minimum inhibitory concentration (MIC) for *Pseudomonas aeruginosa* and *Salmonella typhimurium* was 5 % for methanol and acetone extracts of lemon peel, respectively. The different ability of methanol or acetone extract inhibitory effect may be attributed to the distinct chemical interactions of the two solvents. Regarding the growth inhibitory effects of aqueous extracts, WLP consistently retained the ability to inhibit *E. coli* growth over time for all three concentrations tested. Interestingly, DLP displayed a progressive loss of activity at 12.5 % and 6.25 % of water extract, with some evidence of slightly increased microbial growth, potentially due to the dilution effect of antibacterial compounds combined with the presence of nutrient residues potentially enriching the bacterial culture. This effect may be due to the low concentrations of antimicrobial compounds that were insufficient to exert an inhibitory effect, while other water-soluble constituents (such as simple sugars or soluble amino acids) may be exploited as additional growth substrates from *E. coli*, thus promoting their growth.

The inhibitory properties may primarily be attributed to various compounds such as quercetin, tannic acid, gallic acid, and essential oils of lemon pulp (e.g., limonene), which exhibit different mechanisms of action, including membrane rupture, inhibition of ATPase activity, leakage of essential biomolecules, and enzyme inactivation [49,50]. Limonene is typically the main constituent of the essential oils of *Citrus* × *limon*, accounting for approximately 60 % of the total essential oil content. Its known antimicrobial activity may contribute significantly to the overall inhibitory effects observed in the extracts [51]. However, further studies will be required to deeply characterize the content of essential oils in the considered WLP and DLP by-products.

The differences in extract performance may be attributed to the distinct properties of the solvents used: methanol is a polar solvent that promotes hydrogen bonding, while acetone is a polar aprotic solvent which provides high versatility in solubilizing a broader range of organic compounds.

El Mannoubi et al. [36] indicated that methanol is more effective at extracting low-molecular-weight polyphenols, whereas acetone is particularly efficient for high-molecular-weight flavanols.

Other studies have shown that different extraction methods can significantly influence the antimicrobial activity of phytochemicals. For instance, a study on the antimicrobial effect of various solvent extracts of pomegranate peel found that acetone and methanol extracts exhibited higher antibacterial activity against *E. coli* compared to aqueous extracts [38]. Similarly, Thouri et al. [37] indicated that methanol and acetone extracts had superior antimicrobial properties against *E. coli* compared to water extracts, highlighting the importance of solvent choice in maximizing the yield of bioactive compounds. These data underscore the critical role of solvent selection in maximizing the extraction efficiency of bioactive compounds, with acetone and methanol extracts demonstrating improved inhibitory effects against *E. coli* in comparison to water extracts. Additionally, newer extraction methods like ultrasound-assisted and microwave-assisted extraction can further enhance the yield and selectivity of bioactive compounds [52].

3.4. WLP and DLP extracts supplementation to probiotic strains

The results depicted in the graphs showed in Fig. 5 indicate that the supplementation of WLP and DLP extracts in MRS medium did not highlight any significant differences in the growth of the probiotic strains when compared to the unsupplemented control medium. Both the *L. plantarum* and *L. reuteri* showed similar growth curves, with both strains reaching the growth plateau within a comparable time frame. This suggests that the addition of WLP and DLP extracts does not interfere with the normal growth dynamics of the probiotics' binary fission.

In this study, a possible prebiotic effect of WLP and DLP water extracts were also examined. No significant differences were observed for both treatments tested on *L. plantarum* and *L. reuteri* strains, which suggests that our extracts may effectively inhibit some pathogenic bacteria without negatively affecting lactic acid flora. Notably, the aqueous extracts, despite demonstrating antimicrobial effects at 25 % concentration against O138 *E. coli*, did not impact the growth of lactic acid bacteria strains. This finding is promising, as lactic acid bacteria are generally recognized as beneficial members of the microbiota, contributing to mucosal protection and maintaining a correct eubiosis, while interacting with a multifaceted microbial community. Furthermore, recent studies discussed the potential of lemon pulp as a prebiotic, due to its content of fiber and bioactive compound content [53,54]. However, a more comprehensive evaluation of this prebiotic effect could be achieved by using the whole substrate, as the fiber content in water extracts of DLP may be insufficient to support optimal bacterial growth. Although further studies on various extraction methods and applications for prebiotic effects would be interesting, this result supports the

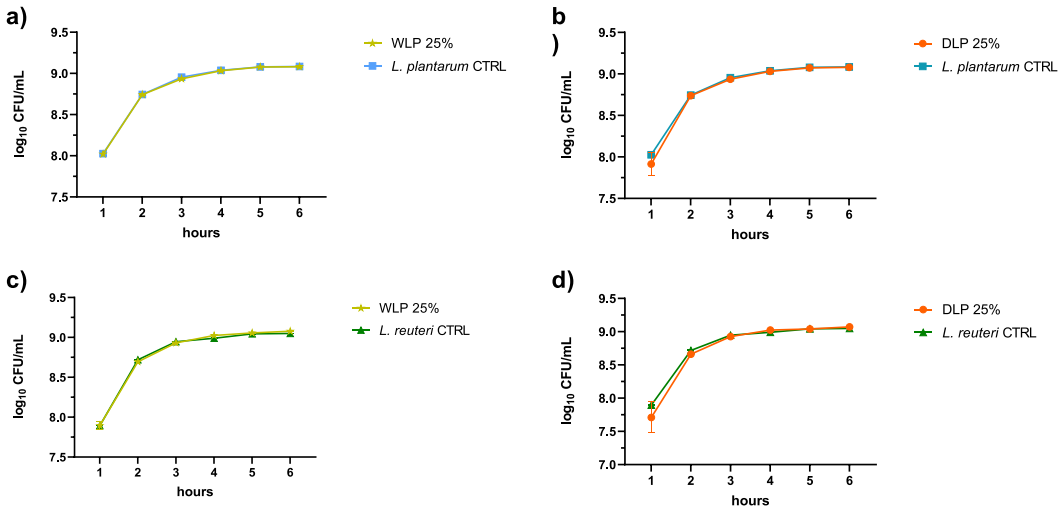

Fig. 5. *L. plantarum* and *L. reuteri* growth with or without supplemented medium with whole lemon pulp (WLP) and depectinized lemon pulp (DLP) water extracts. **a)** 25 % of water extracts of WLP and 25 % of water for *L. plantarum* control. **b)** 25 % of water extracts of DLP and 25 % of water for *L. plantarum* control. **c)** 25 % of water extracts of WLP and 25 % of water for *L. reuteri* control. **d)** 25 % of water extracts of DLP and 25 % of water for *L. reuteri* control.

potential application of lemon by-products in animal nutrition, as they may inhibit pathogenic strains while maintaining a neutral effect on beneficial microorganisms.

The obtained findings are particularly promising for the valorisation of lemon by-products in animal nutrition. Lemon pulp can be considered as a low-cost by-product, priced approximately €0.04/kg for both WLP and DLP, making it a cost-effective ingredient for farmers. Its use offers nutrients supply together with antioxidants and antimicrobial compounds. Prioritizing the use of depectinized pulp, a residual by-product from the pectin industry, aligns with sustainability goals. Whole lemon pulp retains additional value for food production, as its pectin content can be extracted for use in products such as jam, fruit juice, and ice cream. In contrast, DLP represents the final residue of lemon processing. Despite DLP revealed lower antioxidant properties compared to WLP, it aligns with the principles of circular farming with a residual nutritional value and antimicrobial compounds.

In this study, lemon by-products were extracted exclusively for *in vitro* analysis and characterization of functional properties. At farm level, the lemon pulp (whole or depectinized) due to its low cost and simplicity of use, can be supplemented without further processing, maintaining its appeal as a circular and low-cost feed ingredient. *Citrus* by-products, such as those derived from orange, tangerine, and lemon, share similar nutritional profiles and are often combined during industrial processing to form a mixed *Citrus* pomace commonly used in ruminant nutrition. Additionally, farmers frequently include different citrus by-products in animal diets based on seasonal availability and industry supply, as the cost between these by-products is generally negligible. The supplementation of fresh citrus by-products into livestock feed as sources of carbohydrates and fiber represents an environmentally sustainable strategy for reusing industrial waste, with minimal energy input required beyond transport. These by-products can also be stored through drying, ensiling, pelleting, or using as molasses, broadening their applicability in animal nutrition [55]. As Liotta et al. (2019) showed in their trial, the inclusion of dry orange pulp at 10 % on a dry matter basis and lemon molasses at 4 % on a dry matter basis could be a valuable strategy in sheep diet, which could improve milk and cheese nutritional quality. Both whole and depectinized lemon pulp, obtained after industrial processing, could be directly used as feed ingredients. However, it is essential that the material is cooled before consumption and properly stored to prevent percolation and spoilage.

4. Conclusions

Both whole and depectinized lemon pulp represent promising functional by-products, distinguished by their different antioxidant and antimicrobial properties. Although DLP demonstrates lower antioxidant and antimicrobial activity, likely due to the loss of functional compounds during the depectinization process, its residual antibacterial activity combined with its low cost could present a valuable opportunity to valorize this by-product in animal nutrition as a cheap raw material with beneficial health effects. Their utilization within a circular economy and precision nutrition framework could not only enhance animal health but also contribute to the sustainability of livestock production. This study highlights the nutritional and functional potential of whole and depectinized lemon pulp as valuable agro-industrial by-products for animal feeding. Future studies will be necessary to provide a comprehensive metabolomic characterization and investigate their dietary supplementation on animal health and performance, in line with circular economy and One Health principles.

CRediT authorship contribution statement

Cristina Spedale: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation. **Sara Frazzini:** Methodology, Investigation. **Luciana Rossi:** Writing – review & editing, Supervision, Resources, Conceptualization. **Matteo Dell'Anno:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation. **Vincenzo Chiofalo:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

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

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Data availability

Data are available within the manuscript, and the datasets will be made available upoun reasonable request.

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