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Unravelling the immunomodulatory role of apple phenolic rich extracts on human THP-1- derived macrophages using multiplatform metabolomics

Noelia Cambeiro-Pérez [a], María Figueiredo-González [a], María Rosa Pérez-Gregorio [b,*], Catarina Bessa-Pereira [b], Víctor De Freitas [b], Borja Sánchez [c], Elena Martínez-Carballo [a,*]

[a] Food and Health Omics, Department of Analytical and Food Chemistry, Faculty of Sciences, Campus da Auga, University of Vigo, 32004 Ourense, Spain
[b] LAQV/REQUIMTE, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, E-4169-007 Porto, Portugal
[c] Department of Microbiology and Biochemistry, Dairy Research Institute of Asturias, Spanish National Research Council (IPLA-CSIC), Paseo Río Linares sn. 33300, Villaviciosa, Asturias, Spain

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ABSTRACT

Apples represent a significant source of dietary phenolic compounds with evidenced anti-inflammatory and immunomodulatory activities. Nevertheless, the effect of the whole apple matrix on human macrophages is unknown. In this context, our study attempts to evaluate the effect of apple-derived phenolic compounds-rich extracts (pulp, peel and leaf) on IL-1β production in THP-1-differentiated macrophages and derived metabolic alterations through untargeted metabolomics. Our results have showed that apple pulp treatment inhibited the release of the pro-inflammatory cytokine IL-1β induced by LPS in THP-1 macrophages by ELISA analysis. Metabolomics demonstrate that different proportions of phenolic compounds led to differential alterations in the metabolism of THP-1 macrophages. Indeed, apple extracts promoted alterations in lipid, carbohydrate, amino acid and vitamins as well as cofactors metabolism. Specifically, leaf extracts were characterized by alteration of galactose metabolism while the extracts derived from the fruit showed predominant alterations in lipids metabolism. All extracts mimicked the response observed under normal conditions in LPS-stimulated macrophages, inhibiting LPS response. Thus, the phenolic enriched extracts from apples will be a good source of natural compounds with a beneficial effect against inflammation, and they may be applied as a food supplement and/or functional ingredient for the treatment of inflammatory diseases.

1. Introduction

Apple constituents have evidenced immunomodulatory effects through different mechanisms such as antioxidant potential, anti-inflammatory processes, modulation of signal transduction pathways, and anti-proliferative and apoptosis-inducing activity (Gerhauser, 2008). The effect of apple phenolic compounds, as the most consumed fruit in the western diet (Scalbert & Williamson, 2000), on the immune system could be a therapeutic tool for modulating the immune response and managing inflammatory diseases.

Apple phenolic content depends on several factors such as variety, maturity stage, growing region and agricultural practices (Napolitano et al., 2004; Petkovsek, Slatnar, Stampar, & Veberic, 2010; Renard, Dupont, & Guillermin, 2007; Van der Sluis, Dekker, De Jager, & Jongen, 2001). However, the peel is generally the richest part of the fruit in terms of polyphenols (Veberic et al., 2005). Likewise, apple leaves are considered a valuable agro-industrial by-product due to their richness in phenolic compounds (Bonarska-Kujawa, Cyboran, Oszmianski, & Kleszczynska, 2011; Liaudanskas, Viškelis, Raudonis, et al., 2014; Petkovsek et al., 2010; Sowa et al., 2016; Walia, Kumar, & Agnihotri, 2016).

The anti-inflammatory and immunomodulatory activity of apple phenolic compounds have attracted huge attention last years. There is evidence that apple extracts are capable of modulating the expression of several pro-inflammatory genes. They demonstrated the ability to inhibit the expression of relevant enzymes such as nitric oxide synthase (iNOS) that leads to a reduction in nitric oxide (NO) production, cyclooxygenase-2 (COX-2) that leads to a reduction in prostaglandin E2 (PGE2) synthesis and the cytochrome P450 3A4 (CYP3A4). They also modulate the immune system through the modification of cytokine production, decreasing pro-inflammatory cytokines levels of TNF-α, IL-1β, IL-6, IL-8, CXCL9 and CXCL10 in both *in vivo* (Wistar rats) and *in vitro* models (Raw 264.7, DLD-1, T84, MonoMac6, Jurkat, A549 cell lines).

* Corresponding authors.
E-mail addresses: maria.gregorio@fc.up.pt (M.R. Pérez-Gregorio), elena.martinez@uvigo.es (E. Martínez-Carballo).

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Moreover, apple extracts are able to suppress the inflammatory response through inhibition of NF-kB signaling pathways and transcription factors STAT1 and IRF1 (Cho & Yoon, 2012; Fathy & Drees, 2015; Jung, Triebel, Anke, Richling, & Erkel, 2009; Lauren et al., 2009; Lee et al., 2020; Yousefi-Manesh et al., 2019).

Untargeted metabolomics constitutes a powerful tool for biological interpretation of molecular and metabolic changes induced by bioactive compounds in human immune cells (Cambeiro-Pérez et al., 2021, 2018, 2020). The present study aims to reveal metabolic signatures associated with the anti-inflammatory mechanisms triggered by apple extracts in THP-1, a human monocytic leukaemia cell line, which in the presence of phorbol esters, can differentiate into macrophage-like cells, providing an useful model for exploring diverse mechanisms associated with macrophages regulation (Auwerx, 1991).

An untargeted multiplatform metabolite profiling by LC-MS and GC–MS was conducted to characterize THP-1-derived macrophages phenotypes under apple extracts influence and the anti-inflammatory mechanisms triggered by apples in LPS-stimulated cells. The inhibitory activity of apple extracts in THP-1-derived macrophages with particular emphasized on the secretion of a key inflammatory mediator, IL-1β, was also evaluated.

2. Material and methods

2.1. Chemicals and solutions

The human monocytic cell line THP-1 was obtained from the European Collection of Authenticated Cell Cultures (ECACC) (#88081201) and routinely cultured in RPM1 1640 medium containing L-alanyl-glutamine and sodium bicarbonate (Sigma Aldrich, Madrid, España) and supplemented with 10 % fetal bovine serum (FBS) (ICN Flow; Costa Mesa, CA, USA) and 1.0 % of an antibiotic/antimycotic solution containing 100 U mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin and 0.25 μg mL^{-1} of amphotericin B from Sigma Aldrich. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), dimethyl sulfoxide (DMSO), Lipopolysaccharides (LPS) from *Escherichia coli* O111:B4 purified by phenol extraction and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Aldrich.

Phenolic compounds standards: (−)- Epicatechin, (+)- Catechin, Malvidin 3-*O*-glucoside, Procyanidin B1 and Procyanidin B2 were purchased from Extrasynthese (Genay, France); and Chlorogenic acid, Phloretin, Phloridzin dehydrate and Quercetin from Sigma Aldrich. The individual stock solutions of each phenolic compound standard were prepared in MeOH using 10 mg L^{-1} mixes of these individual stocks and were stored in amber flasks at −20 °C.

Surrogate standards (DL-Norvaline, Succinic acid-2,2,3,3-d_4 and *trans*-Cinnamic-d_7 acid) were purchased from Sigma Aldrich and prepared at 100 mM in water or methanol depending on their solubility. The surrogate mix solution was prepared in methanol at 1.0 mM. Internal standards (PCB30 and PCB204) were acquired from Accustandard (New Haven, USA). FAME mix solution and saturated Alkane Mixture (C7–C40) were acquired from Supelco (Bellefonte, USA) and were prepared in acetone at 1.0 mg L^{-1}. For derivatization: N-Methyl-N-trimethylsilyl trifluoroacetamide (MSTFA), chlorotrimethylsilane (TMCS) and pyridine were purchased from Sigma Aldrich; and Methoxylamine hydrochloride (MeOX) was from Supelco.

The IL-1β level in cell culture supernatants was measured by using the Human IL-1 beta/IL-1F2 DuoSet ELISA kit from R&D Systems following the manufacturer's instructions.

2.2. Plant materials

Leaves and fruits of *Malus domestica* were harvested in September 2017 in Castro de Beiro (42°2′48.7″N 7°5′36.0″W), Ourense (NW, Spain). Apple fruits were grown under organic agricultural practices. Apple leaves were dried between filter paper at room temperature and then ground to powder by using a crusher. Fruits were stored at 4.0 °C until use no >1 week. Then, apples were mechanically peeled and cored, and sprayed with an aqueous formic acid (3.0 %, v/v) solution in order to minimize polyphenol oxidation. Cored apples, peel and pulp were frozen separately at −80 °C, freeze-dried and ground to fine powder.

2.3. Phenolic compounds extraction

The extraction of phenolic compounds was processed as proposed by Pérez-Gregorio, García-Falcón, Simal-Gándara, Rodrigues, & Almeida, (2010). An amount of 0.50 g of each sample (dry-powdered leaves and freeze-dried cored apples, peel and pulp) was extracted two times with 20 mL and last with 10 mL of MeOH:H_2O (8:2, v/v) solution. Each extraction cycle was followed by ultra-sonication for 40 min and centrifugation at 3600 rpm during 10 min. A purification step with Strata C18-E cartridges (Phenomenex, Spain) was also included to clean-up to plysaccharides prior to analysis enhancing the purity of phenolic compounds, according to manufacturer specifications. Supernatants were, on the one hand, evaporated to dryness under a gentle nitrogen stream, redissolved in 1.0 mL of MeOH:H_2O (9:1, v/v), passed through hydrophilic PTFE (0.22 μm) syringe filters and transferred to vials to its characterization and quantification. On the other hand, methanolic extracts were evaporated to 2.0 mL and redissolved in 20 mL H_2O (1:10, v/v) for subsequent freeze-drying and performance of the *in vitro* assay.

2.4. Apple phenolic compounds characterization

2.4.1. Untargeted phenolic compounds profiling

Firstly, an untargeted phenolic profile of apple samples was performed on a LTQ-Orbitrap XL mass spectrometer coupled with an Accela HPLC system equipped with a Kinetex™ 2.6 μm C18 (100 Å, LC Column 100 × 2.1 mm) column. The injection volume was 20 μL. The MS instrument, operating in negative mode, was set at 3.1 kV of spray voltage, 30 V of heated capillary voltage, 280 °C of capillary temperature and the tube lens was established at 100 V. Molecular ions fragmentation was achieved by collision-induced dissociation (CID) applying 35 V. All MS and MS/MS spectra were acquired in the data-dependent mode. The instrument executed one MS scan followed by an MS/MS scan of each one of the three most intense peaks.

The mobile phases for LC separation were (A) 0.50 % (v/v) HCOOH in H_2O and (B) MeOH. Those were pumped at 0.25 mL min^{-1} in the following gradient: 15 % B during 2.0 min, changing to 70 % B in 23 min, and then to 100 % B in 10 min, changing to initial conditions in 5.0 min and holding them for another 5.0 min, giving a total analysis time of 45 min.

2.4.2. Targeted phenolic compounds profiling

The targeted phenolics characterization of apple samples were performed on a TSQ Quantum Discovery triple stage quadrupole mass spectrometer equipped to an electrospray interface from Thermo Fisher Scientific. The MS was coupled with a Dionex Ultimate 3000 HPLC system, which contains a quaternary pump, auto sampler, degasser as well as column compartment, interfaced to a PC computer running the Thermo Scientific™ Dionex™ Chromeleon™ CDS. Thermo Scientific Xcalibur processing and instrument control software was used. Chromatographic separations were performed with a Kinetex™ 2.6 μm C18 (100 Å, LC Column 100 × 2.1 mm) column. The mobile phases for LC separation were (A) 0.50 % (v/v) HCOOH in H_2O and (B) MeOH. The temperature of the LC column was kept constant at 25 °C. The gradient was: 95 % during 2.0 min change to 95 % B in 25 min, changing to 45 % B and 50 % C in 3.0 min and hold for 0.10 min, changing to initial conditions in 7.0 min. The injection volume was set to 2.0 μL by LC flow rate of 0.25 mL min^{-1}. MS/MS analysis was performed using argon as the collision gas and nitrogen as the nebulizer gas. Identification was performed using selected reaction monitoring (SRM) in negative mode). Capillary voltage was set to 4.5 kV. Capillary temperature of 275 °C,

sheath and auxiliary gas pressure of 45 and 5.0 psi respectively were selected.

2.4.3. Method validation of the targeted phenolic compounds profiling

The analytical method was in-house validated in terms of linearity, precision, trueness/accuracy, limits of detection and quantification (LODs and LOQs) and uncertainly values.

External linear calibration was used to quantify the targeted phenolic compounds in the selected apple samples. Flavan-3-ols were quantified with their respective calibration curves ((+)- catechin, (−)- epicatechin, procyanidin B1 and procyanidin B2); flavonols by using quercetin calibration curve; dihydrochalcones using phlorizin and phloretin calibration curves; phenolic acids by using chlorogenic acid calibration curve and anthocyanins with malvidin-3-O-glucoside calibration curve. Linear calibration curves fit reasonably ($R^2 > 0.999$) in a twelve-point calibration curve with a concentration scale of two or three orders of magnitude, depending on the compound (50–10,000 ng g^{-1}).

LODs and LOQs were evaluated on the basis of the noise obtained with the analysis of unfortified samples (n = 4). They are defined as the concentration of the analyte that produced a signal-to-noise ratio of 3 and 10, respectively and were then tested experimentally by spiking five replicates of blank samples at such levels. The results showed LODs of 3.4, 0.50, 1.5, 0.30 and 0.25 mg kg^{-1} for flavan-3-ols, chlorogenic acid, malvidin-3-*O*-glucoside, quercetin, and dihydrochalcones, respectively and LOQs of 11, 1.3, 4.6, 0.90 and 0.75 mg kg^{-1} for the same compounds. To verify the limits for real samples (e.g., effects of ion suppression), signal-to-noise ratios for the analytes in extracts samples in which concentrations were close to the calculated LOQs were determined. Results obtained for the accuracy were in the range from 1.0 to 17 %. The comparison between peak areas, between extracted blank samples and pure diluted substances showed<9.0 % signal suppression.

2.5. In vitro assay and untargeted metabolomics

2.5.1. THP-1 co-cultivation

According to our previous metabolomics study (Cambeiro-Pérez et al., 2021), THP-1 cells were cultivated into 12-well microplates (2.0×10^6 cells mL^{-1}) and subsequently differentiated into mature macrophage-like cells by stimulating with 25 nM TPA for 48 h. After differentiation, cells were stimulated in culture with apple extracts (leaf, apple, peel and pulp) at 1.0 mg mL^{-1}, with and without LPS (1.0 μg mL^{-1}). In addition, untreated and LPS-stimulated THP-1-derived macrophages (without the presence of the extracts) were also assayed as negative (BASAL) and positive (LPS) control, respectively. Blank samples (with no cells) were also included in the study. All conditions were tested in triplicate. The microplates were incubated for 2 days at 37 °C with 5.0 % CO_2. Cell viability, performed by MTT assay, was not significantly altered by the doses of treatments used in this study.

2.5.2. Cytokine measurement assay

Cell culture supernatants from THP-1-differentiated macrophages were collected, in triplicates, after 48 h of stimulation with both LPS and extracts, and stored at –80 °C until assayed for cytokines. Supernatants of control cells (negative and positive) were also collected and analyzed. The level of IL-1β was quantitatively determined using the enzyme-linked immunosorbent assay (ELISA) kit Human IL-1 beta/IL-1F2 DuoSet ELISA (R&D Systems), according to manufacturer's instructions.

2.5.3. Untargeted metabolomics

Quenching samples were obtained as was described before (Cambeiro-Pérez et al., 2021), and stored to − 80 °C until injection by LC-HRMS and derivatization prior to injection by GC-QqQ-MS. Quality Control (QC) samples have also been employed for controlling the quality of metabolomics data, by pooling 45 μL from each of analyzed samples. In order to cover the metabolome as broadly as possible, samples were analyzed following a multiplatform approach (LC-HRMS and GC-QqQ-MS) as was previously described in detail (Cambeiro-Pérez et al., 2021).

2.6. Data analysis and statistics

The results of cytokine measurement assay were expressed as mean values ± standard deviations (SD) of the three independent experiments. Statistical significance of differences between the mean values were calculated using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test ($p < 0.05$). All analyses were performed using SPSS 23.0 software.

After sample acquisition of untargeted metabolomics, a differential data processing protocol was carried out depending on the analytical platform employed. For LC-MS samples, MassHunter Profinder Software B.08.00 (Agilent Technologies), for deconvolution, followed by Mass Profiler Professional (MPP) B.14.08 (Agilent Technologies), for filtering, alignment and identification; were employed. In the case of GC–MS samples, they were processed by MassHunter Unknowns Analysis Tool B.07.01 (Agilent Technologies) for deconvolution and identification, followed also by Mass Profiler Professional (MPP) B.14.08 (Agilent Technologies) for filtering and alignment. Entities list were exported for both LC and GC from MPP after filtering and normalization to create a combined entity list for further multivariate statistical analysis. Finally, biological interpretation was performed by MetaboAnalyst 4.0 (http://www.metaboanalysts.ca) through pathway analysis tool and the metabolite set library Homo sapiens (KEGG) based on normal human metabolic pathways. Moreover, other freely available online databases were employed as the Human Metabolome database (HMDB; https://hmdb.ca/), KEGG database (http://www.genome.jp/kegg) and LIPID MAPS® (https://www.lipidmaps.org/).

3. Results and discussion

3.1. Phenolic composition of apple phenolic compounds-rich extracts

Tentative characterization of phenolic compounds was conducted based on the elution order, the interpretation of their mass spectrum provided by the LC-LTQ-Orbitrap XL-MS, and the information previously reported in the literature. Fig. 1 shows the resulting total ion chromatograms (TIC) of different apple extracts: leaf (A), peel (B) and pulp (C).

The characterized phenolic compounds in peel, pulp and leaves of apple extracts, are summarized in Table 1. A total of 18 compounds belonging to different phenolic families were tentatively identified based on the information generated by the LC-HRMS analytical technique such as retention time, observed *m/z* ($[M-H]^-$), calculated *m/z* ($[M-H]^-$), molecular formula, error and fragments produced by the ionization conditions.

Three derivatives of phenolic acids (PA) were detected in apple samples:

- PA1, proposed as *chlorogenic acid* (353.0884 *m/z*; 6.16 min) with a predominant fragment at 191 *m/z,* was the main phenolic acid found in pulp and peel samples but not detected in leaf samples.
- PA2, proposed as *chlorogenic acid isomer* (353.0887 *m/z*; 7.03 min) with a predominant fragment also at 191 *m/z,* was only found in pulp samples.
- PA3, proposed as *p-coumaroyl quinic acid* (337.0926 *m/z*; 8.37 min) with two predominant fragments at 163 and 173 *m/z*, which was found only in peel and pulp samples.

Flavonols (F) were also present in the selected samples. Quercetin glycosides were found in all matrices whilst kaempferol glycosides were only detected in leaf:

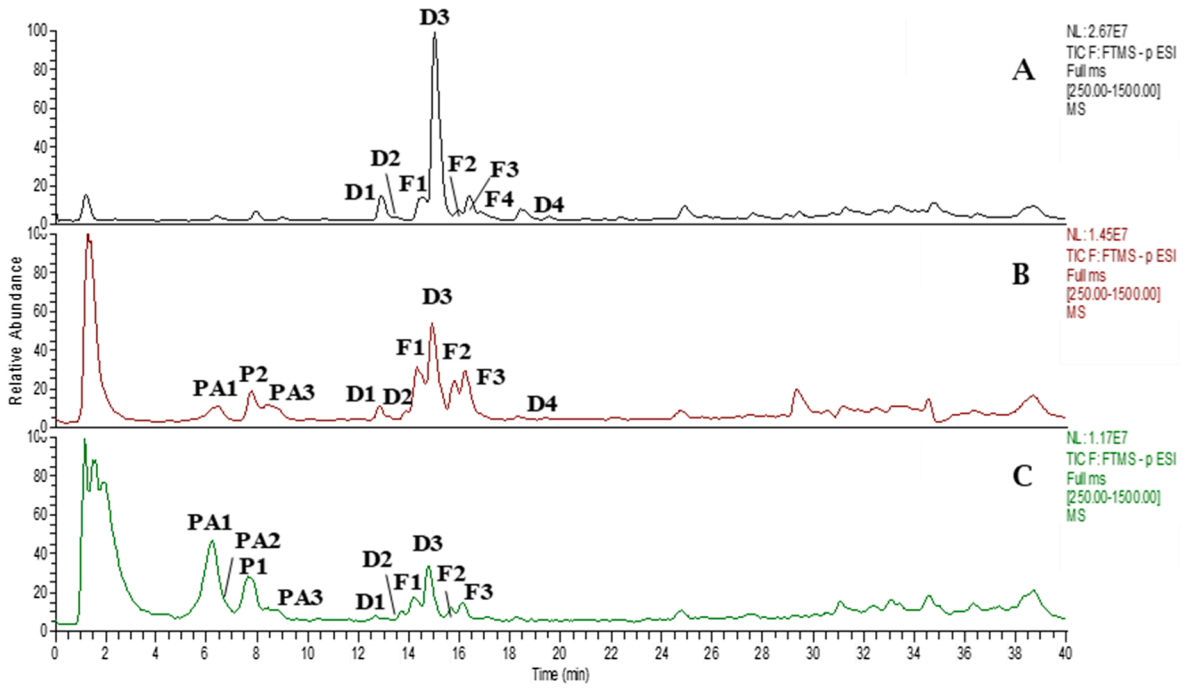

Fig. 1. Total ion chromatograms (TIC) of apple extracts: (A) leaf, (B) peel and (C) pulp obtained by LC-LTQ-Orbitrap XL-MS.

- F1 was tentatively identified as *quercetin-hexoside* (463.0883 *m/z*; 14.1 min) due to the presence of the characteristic quercetin aglycone fragment 301 *m/z*, which could lead to the loss of a hexose moiety (162 *m/z*) from the molecular ion structure.
- F2 was also tentatively identified as a quercetin glycoside because of the 301 *m/z* fragment, which could be identified as *quercetin-pentoside* (433.0774 *m/z*; 15.6 min) due to the possible loss of the pentose unit (132 *m/z*).
- F3, related to quercetin group was proposed as *quercetin-rhamnoside* (447.00932 *m/z*; 16.12 min), as it seems to show the loss of 146 *m/z* from a rhamnose moiety.
- F4 and F5 were tentatively identified as *kaempferol-hexoside* (447.0942 *m/z*; 16.39 min) and *kaempferol-pentoside* (417.0834 *m/z*; 18.89 min), for the characteristic aglycone fragment 285 *m/z* and the loss of the hexose unit 162 *m/z* (F4) and of the 285 *m/z* (F5) from the molecular structure.

Four Dihydrochalcones (D) were detected in apple samples:

- D1 was proposed as *3-hydroxyphlorizin* (451.1244 *m/z*; 12.68 min) with a fragment at 289 *m/z* indicates a loss of the glucoside moiety, as well as the measured mass of the molecular ion (M + .) at *m/z* 451.1244 is consistent with the expected molecular mass for *phlorizin* (435.1297 *m/z*) plus a hydroxyl group (16 *m/z*) in the structure.
- D2 was proposed as *phloretin-xyloglucoside* (567.1715 *m/z*; 13.65 min). The fragments at 273 and 167 *m/z* indicate loss of the pentoside (132 *m/z*) and the hexoside (162 *m/z*) moiety, respectively.
- D3 was tentatively identified as *phloretin-hexoside* or *phlorizin* (435.1297 *m/z*; 14.75 min). The fragment 273 *m/z* indicates a loss of the glucoside moiety (162 *m/z*). D4 was proposed as the aglycone *phloretin* (273.0761 *m/z*; 19.4 min) with the predominant fragment 167 *m/z*. Phloretin was only detected in apple leaves and peel whilst the rest of dihydrochalcones were present in all the apple extracts.

Four derivatives of flavan-3-ols (P) were also found in apples, both in monomeric (*catechin* and *epicatechin*) and oligomeric forms (*procyanidin B1* and *procyanidin B2*):

- P1 was proposed as *catechin* (289.0712 *m/z*; 4.46 min) and it was found in peel and pulp samples.
- P2 proposed as *epicatechin* (289.0711 *m/z*; 7.59 min), was found in leaf and pulp with predominant fragments at 271, 245 and 179 *m/z*. Dimeric forms were found in all matrices:
- P3 proposed as *procyanidin B1* (577.1342 *m/z*; 6.04 min), and P4 proposed as *procyanidin B2* (577.1340 *m/z*; 8.62 min), exhibited the main fragment at 289 *m/z* that could be due to the loss of one catechin/epicatechin unit, besides the fragment 407 *m/z*.

Two cyanidin (C) derivatives were tentatively identified based on their main fragment at 285 *m/z*:

- C1 was proposed as *cyanidin-hexoside* (44.0822 *m/z*; 8.58 min) indicates a loss of the hexose moiety (162 *m/z*).
- C2 was proposed as *cyanidin-pentoside* (417.0825 *m/z*; 9.82 min) indicates a loss of the pentose moiety (132 *m/z*).

Phenolic compounds characterization in both apple fruit and leaves has been widely described previously (Alonso-Salces et al., 2004; de Bernonville et al., 2011; Çam & Aaby, 2010; Ceymann et al., 2011; De Paepe et al., 2013; Jakobek, García-Villalba, & Tomás-Barberán, 2013; Lamperi et al., 2008; Li et al., 2014; Liaudanskas, Viškelis, Raudonis, et al., 2014; Mari et al., 2010; Montero, Herrero, Ibáñez, & Cifuentes, 2013; Ramirez-Ambrosi et al., 2013; Reis, Rai, & Abu-Ghannam, 2012; Sommella et al., 2015; Tsao, Yang, Young, & Zhu, 2003; Vrhovsek, Rigo, Tonon, & Mattivi, 2004; Wojdyło, Oszmiański, & Laskowski, 2008; Xiao et al., 2017), some of them provided also exact mass information (De Paepe et al., 2013; Jakobek et al., 2013; Li et al., 2014; Ramirez-Ambrosi et al., 2013; Reis et al., 2012; Sommella et al., 2015).

Individual and total phenolic compound levels of apple extracts, calculated per kilogram of dry weight (DW), were presented in Table 2. As it shown, we found that apple leaves are much better source of total phenolic compounds (26,199 mg kg^{-1}) than peel (3292 mg kg^{-1}) and pulp (2086 mg kg^{-1}), tendency which is consistent with previous studies (Jakobek et al., 2013; Kalinowska, Bielawska, Lewandowska-Siwkiewicz, Priebe, & Lewandowski, 2014; Petkovsek et al., 2010).

Phenolic acids were the predominant phenolic compounds in apple

Table 1
Proposed phenolic compounds in apple extracts.

	Assignment	RT (min)	Observed *m/z* ([M−H]⁻)	Calculated *m/z* ([M−H]⁻)	Molecular formula	Error (ppm)	Fragments *m/z*	References
	Phenolic acids (PA)							
PA1	Chlorogenic acid	6.16	353.0884	353.0877	$C_{16}H_{18}O_9$	1.98	191	Kviklys et al., 2014; Liaudanskas, Viškelis, Jakštas, et al., 2014; Liaudanskas, Viškelis, Raudonis, et al., 2014; Sommella et al., 2015
PA2	Chlorogenic acid isomer	7.03	353.0887	353.0877	$C_{16}H_{18}O_9$	2.83	191	Jakobek et al., 2013; Ramirez-Ambrosi et al., 2013; Sommella et al., 2015
PA3	*p*-coumaroyl quinic acid	8.37	337.0926	337.0930	$C_{16}H_{18}O_8$	−1.18	173, 163	Díaz-García, Obón, Castellar, Collado, & Alacid, 2013; Fromm et al., 2013; Jakobek et al., 2013; Ramirez-Ambrosi et al., 2013; Sommella et al., 2015
	Flavonols (F)							
F1	Quercetin-hexoside	14.10	463.0883	463.0882	$C_{21}H_{20}O_{12}$	0.21	301	Kviklys et al., 2014; Li et al., 2014; Liaudanskas, Viškelis, Jakštas, et al., 2014; Liaudanskas, Viškelis, Raudonis, et al., 2014; Sommella et al., 2015
F2	Quercetin-pentoside	15.60	433.0774	433.0776	$C_{20}H_{18}O_{11}$	−0.46	301	Kviklys et al., 2014; Liaudanskas, Viškelis, Jakštas, et al., 2014; Sommella et al., 2015
F3	Quercetin-rhamnoside	16.12	447.0932	447.0933	$C_{21}H_{20}O_{11}$	−0.22	301	Kviklys et al., 2014; Liaudanskas, Viškelis, Jakštas, et al., 2014; Liaudanskas, Viškelis, Raudonis, et al., 2014; Sommella et al., 2015
F4	Kaempferol-hexoside	16.39	447.0942	447.0933	$C_{21}H_{20}O_{11}$	2.01	285	Li et al., 2014; Vrhovsek et al., 2012
F5	Kaempferol-pentoside	18.89	417.0834	417.0827	$C_{20}H_{18}O_{10}$	1.67	285	Jakobek et al., 2013; Sommella et al., 2015
	Dihydrochalcones (D)							
D1	3-Hydroxyphlorizin	12.68	451.1244	451.1246	$C_{21}H_{24}O_{11}$	−0.44	289, 421	Fromm et al., 2013; Li et al., 2014; Ramirez-Ambrosi et al., 2013; Xiao et al., 2017
D2	Phloretin-xyloglucoside	13.65	567.1715	567.1719	$C_{26}H_{32}O_{14}$	−0.70	273, 167	De Paepe et al., 2013; Fromm et al., 2013; Jakobek et al., 2013; Ramirez-Ambrosi et al., 2013; Sommella et al., 2015; Xiao et al., 2017
D3	Phlorizin	14.75	435.1297	435.1296	$C_{21}H_{24}O_{10}$	0.22	273	Li et al., 2014; Liaudanskas, Viškelis, Jakštas, et al., 2014; Liaudanskas, Viškelis, Raudonis, et al., 2014; Sommella et al., 2015
D4	Phloretin	19.40	273.0761	273.0768	$C_{15}H_{14}O_5$	−2.56	167	De Paepe et al., 2013; Li et al., 2014; Liaudanskas, Viškelis, Raudonis, et al., 2014; Ramirez-Ambrosi et al., 2013
	Flavan-3-ols (P)							
P1	Catechin	4.46	289.0712	289.0717	$C_{15}H_{14}O_6$	−1.72	271, 245, 179	Kviklys et al., 2014; Liaudanskas, Viškelis, Jakštas, et al., 2014; Liaudanskas, Viškelis, Raudonis, et al., 2014; Sommella et al., 2015
P2	Epicatechin	7.59	289.0711	289.0717	$C_{15}H_{14}O_6$	−2.07	271, 245, 179	Kviklys et al., 2014; Liaudanskas, Viškelis, Jakštas, et al., 2014; Liaudanskas, Viškelis, Raudonis, et al., 2014; Sommella et al., 2015
P3	Procyanidin B1	6.04	577.1342	577.1351	$C_{30}H_{26}O_{12}$	−1.55	407, 289	Kviklys et al., 2014; Liaudanskas, Viškelis, Jakštas, et al., 2014; Sommella et al., 2015
P4	Procyanidin B2	8.62	577.1340	577.1351	$C_{30}H_{26}O_{12}$	−1.90	407, 289	Kviklys et al., 2014; Liaudanskas, Viškelis, Jakštas, et al., 2014; Sommella et al., 2015
	Anthocyanins (C)							
C1	Cyanidin-hexoside	8.58	447.0822	447.0933	$C_{21}H_{20}O_{11}$	−2.46	285	De Paepe et al., 2013; Jakobek et al., 2013; Ramirez-Ambrosi et al., 2013; Sommella et al., 2015
C2	Cyanidin-pentoside	9.82	417.0825	417.0827	$C_{20}H_{18}O_{10}$	−0.47	285	Jakobek et al., 2013; Vrhovsek et al., 2004

pulp (1145 mg kg^{-1}), followed by peel (425 mg kg^{-1}) and leaves (101 mg kg^{-1}) as it was previously reported in many apple varieties (Jakobek et al., 2013; Khanizadeh et al., 2008; Tsao et al., 2003; Wojdyło et al., 2008). Chlorogenic acid was the major phenolic acid and it was found in leaf (100 %), peel (86 %) and pulp samples (78 %). Other hydroxycinnamic acids such as chlorogenic acid isomer (12 % in peel and 8.2 % in pulp) and *p*-coumaroyl quinic acid (2.2 % in peel and 14 % in pulp) were also found. Several authors have also reported isomers of chlorogenic acid in apples (Jakobek et al., 2013; Ramirez-Ambrosi et al., 2013; Sommella et al., 2015; Vrhovsek et al., 2012) and other authors have also detected higher abundance of chlorogenic acid than *p*-coumaroyl quinic acid (Ceymann et al., 2011; Jakobek et al., 2013; Vrhovsek et al., 2004). Nevertheless, further studies with NMR techniques would help clarifying their isomerism.

Flavonols were the predominant phenolic compounds in leaves (2471 mg kg^{-1}), followed by peel (1165 mg kg^{-1}) and pulp (29 mg kg^{-1}). Glycosylated and aglycone forms of quercetin and kaempferol were found in the selected samples and were confirmed by LC-QqQ methodology according to the literature (Wojdyło et al., 2008). Quercetin derivatives (quercetin-hexoside, quercetin-pentoside I-II and quercetin rhamnoside) represented the major flavonols in all the selected samples (around 97 % flavonols) whilst kaempferol derivatives (kaempferol-hexoside and kaempferol-pentoside I-II) were only detected in leaves and peel samples (arround 3.0 % flavonols). Quercetin aglycone was only present in peels. Quercetin-hexoside was the main compound in apple leaves (1365 mg kg^{-1}) whereas quercetin-pentoside II was in apple peel (445 mg kg^{-1}) and pulp (16 mg kg^{-1}). Kaempferol-hexoside was the major kaempferol contributor only detected in leaf

Table 2
Phenolic compound content in local red apple extracts (mg kg^{-1} dried weight, DW).

Compounds	RT (min)	Transition reactions (*m/z*)	Leaf	Peel	Pulp
Phenolic acids (PA)					
Chlorogenic acid (PA1) *	9.69	353 > 191	–	425	1145
Chlorogenic acid isomer (PA2)	10.51	353 > 191	–	59	120
p-coumaroyl quinic acid (PA3)	10.91	337 > 173	–	11	206
Total phenolic acids			101	495	1471
Flavonols (F)					
Quercetin-hexoside (F1)	15.03	463 > 301	1365	304	3.9
Quercetin-pentoside I (F2)	15.54	433 > 301	166	165	8.1
Quercetin-pentoside II (F2)	15.95	433 > 301	747	445	16
Quercetin-rhamnoside (F3)	16.25	447 > 301	–	232	0.69
Kaempferol-hexoside (F4)	16.32	447 > 285	193	–	–
Kaempferol-pentoside I (F5)	16.89	417 > 285	–	1.9	–
Kaempferol-pentoside II (F5)	17.18	417 > 285	–	5.9	–
Quercetin (F6) *	17.70	301 > 301	–	12	–
Total flavonols			2471	1165	29
Dihydrochalcones (D)					
Hydroxyphlorizin (D1)	13.73	451 > 289	1462	15	7.7
Phloretin-xyloglucoside (D2)	14.40	567 > 273	–	120	52
Phlorizin (D3)*	15.08	435 > 273	19,976	398	83
Phloretin (D4)*	18.05	272 > 167	2168	9.5	–
Total dihydrochalcones			23,605	542	143
Flavan-3-ols (P)					
Catechin (P1) *	8.60	289 > 245	8.4	39	25
Epicatechin (P2) *	10.64	289 > 245	14	25	224
Procyanidin B1 (P3) *	7.96	577 > 407	0.79	82	79
Procyanidin B2 (P4) *	9.62	577 > 407	6.9	0.39	116
Total flavan-3-ols			21	121	444
Anthocyanins (C)					
Cyanidin-hexoside (C1)	11.15	447 > 285	–	693	–
Cyanidin-pentoside I (C2)	12.03	417 > 285	–	29	–
Cyanidin-pentoside II (C2)	13.20	417 > 285	–	246	–
Total anthocyanins			–	968	–
Total polyphenols			26,199	3292	2086

[a]Confirmed with commercial standards.
* Relative standard deviations were <10 % based on three replicates for each sample.

samples with a mean concentration of 193 mg kg^{-1}. Kaempferol-pentoside I-II were only detected in peel with mean concentration levels of 1.9 and 5.9 mg kg^{-1}, respectively. Results showed a general trend and flavonols were found in higher concentration in the peel than the pulp (Jakobek et al., 2013; Lamperi et al., 2008; Tsao et al., 2003). Moreover, kaempferol derivatives were also reported by Jakobek et al. (2013) and Li et al. (2014).

Dihydrochalcones were the polyphenols detected in the highest concentration with the phlorizin as the main component in leaves (19,976 mg kg^{-1}), followed by peel (398 mg kg^{-1}) and pulp (83 mg kg^{-1}). Hydroxyphlorizin was also found in all the selected samples, mainly in leaves (1462 mg kg^{-1}), followed by peel (15 mg kg^{-1}) and pulp (7.7 mg kg^{-1}). Phloretin was determined mainly in leaves (2168 mg kg^{-1}) and peels (9.5 mg kg^{-1}). Phloretin-xyloglucoside was detected in higher concentration in peel (120 mg kg^{-1}) than in pulp (52 mg kg^{-1}). Similar results were previously reported by other authors (Tsao, Yang, Young, & Zhu, 2003; Alonso-Salces et al., 2004; Mari et al., 2010, Jakobek et al., 2013; Jakštas et al., 2014; Kviklys et al., 2014; Li et al., 2014; Vrhovsek, Rigo, Tonon, & Mattivi, 2004; Xiao et al., 2017).

Flavan-3-ol content was higher in pulp (444 mg kg^{-1}) than peel (121 mg kg^{-1}) than in leaves (21 mg kg^{-1}). Among monomeric forms, catechin predominated in peel samples (39 mg kg^{-1}) whilst epicatechin predominated in apple pulp (224 mg kg^{-1}). Dimer's content was also higher in pulp (195 mg kg^{-1}) than in peel (82 mg kg^{-1}) and in leaf (7.7 mg kg^{-1}). These results were consistent with the results reported by other authors (Jakobek et al., 2013). Regarding to proanthocyanidin content it was difficult to compare results with the literature due to the lack of detailed quantitative information and the different methodologies performed.

Anthocyanins were present only in red apple peel (967 mg kg^{-1}) as three cyanidin glycoside derivatives: cyanidin-hexoside (693 mg kg^{-1}), and cyanidin-pentoside isomers, I and II, in less amount (29 and 246 mg kg^{-1}, respectively). These results were consistent with the results reported by other authors (Alonso-Salces et al., 2004; de Bernonville et al., 2011; Ceymann et al., 2011; De Paepe et al., 2013; Lamperi et al., 2008; Milani et al., 2015; Montero et al., 2013; Petkovsek et al., 2010; Ramirez-Ambrosi et al., 2013; Sobhani, Farzaei, Kiani, & Khodarahmi, 2020; Vrhovsek et al., 2012; Zhang et al., 2020).

3.2. Effect of apple phenolic rich extracts on IL-1β production in THP-1 macrophages

To evaluate the potential of apple extracts (apple, peel and pulp) in regulating IL-1β production by THP-1-differentiated macrophages stimulated with LPS; cells were simultaneously exposed to LPS and apple extracts (1 mg/ml) for 48 h h. Subsequently, cell culture supernatants of each treatment were collected and the IL-1β level was quantified by ELISA.

As shown in Fig. 2, the level of IL-1β was markedly increased in culture supernatant upon treatment with LPS, which was only significantly inhibited by pulp extract. Inhibition was also observed by apple extracts ($p < 0.0541$), although it was not significant for a 95 % confidence level.

Despite evidences of the anti-inflammatory potential of apple phenolic compounds and apple extracts, the role of the fruit in regulating the IL-1β production in human macrophages stimulated with LPS has not been described in the past. Evidence suggests, that when macrophages are stimulated with LPS, there is a markedly increased in pro-inflammatory cytokine production, a typical feature of various inflammatory diseases (Sharif, Bolshakov, Raines, Newham, & Perkins, 2007). Accordingly, as THP-1 cells are highly sensitive to LPS and respond by expressing several inflammatory cytokines, in this study, LPS-stimulated THP-1-derived macrophages were used as an *in vitro* model of inflammation.

Our results showed that apple pulp effectively inhibited the production of IL-1β on LPS-treated macrophages. These results indicated the anti-inflammatory potential of apple pulp in LPS-induced inflammation. Since IL-1β is a central inflammatory mediator, our findings therefore suggested that apples are a promising therapeutic agent for the treatment of inflammation.

3.3. Immunomodulatory role of apple phenolic compounds-rich extracts in THP-1 macrophages metabolome

A total of 507 entities were found to be differentially regulated (p-value < 0.050 and fold change > 2.0) between conditions: untreated stimulated THP-1 macrophages without LPS (Basal) and with LPS (LPS),

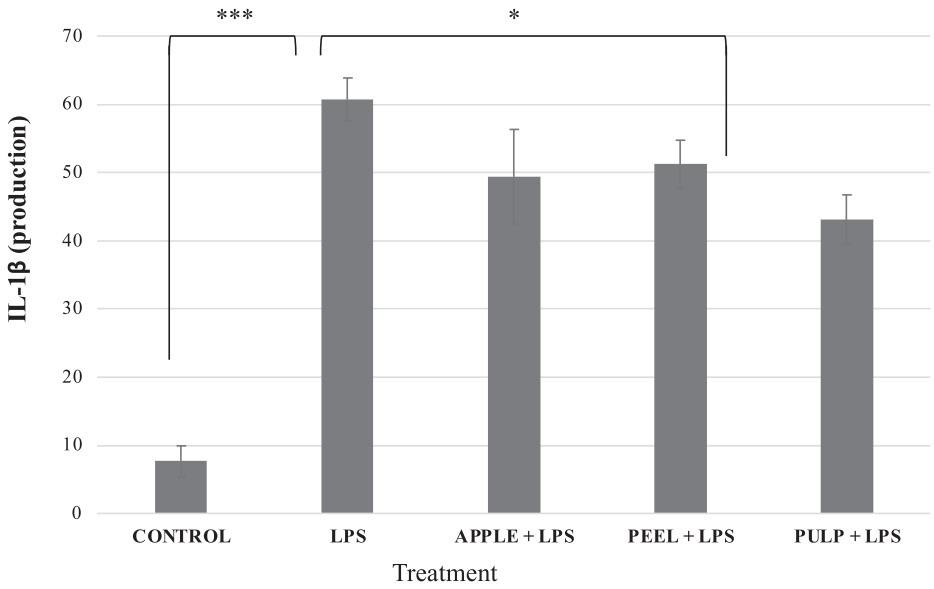

Fig. 2. Inhibitory effects of apple extracts on IL-1β production in LPS treated THP-1 macrophages. THP-1 macrophages co-treated with apple extracts (100 μM) and the pro-inflammatory molecule LPS (uM) for X h and the levels of IL-1β were measured by sandwich ELISA. Values are represented as mean ± SD of at least three independent experiments. *** $p < 0.001$ compared with the unstimulated THP-1 macrophages. * $p < 0.05$ compared with LPS-treated THP-1 macrophages.

treated stimulated THP-1 macrophages with apple extracts (Leaf, Apple, Peel and Pulp) and treated stimulated THP-1 macrophages with apple extracts and LPS (Leaf_L, Apple_L, Peel_L, Pulp_L), Blank and QC.

A PLS-DA was carried out to have a complete overview of the analytical methodology quality with 47 % of the explained variance with the three first principal components (accuracy of the model of 90.538 %, R2 = 0.348 and Q2 = 0.031) (Fig. 3A). Data showed a grouped distribution without outlier samples, which means that stability and repeatability were maintained throughout the sequence. In addition, as expected, blank and QC were clustered separately from the rest of conditions. To analyze the possible relationships between conditions, a hierarchical clustering analysis by applying Pearson's uncentered similarity measure and complete linkage was performed (Fig. 3B). The results suggested the existence of two main clusters, one of them constituted by the extracts, both in the presence and absence of LPS, and a second cluster represented by the control conditions (Basal, LPS, Blank and QC). Furthermore, at a second level of the hierarchical clustering, there seems to be a similar behavior between the following pairs of conditions: Apple-Pulp and Leaf-Peel.

Although both PLS-DA and clustering revealed a differential behavior between treated and untreated cells, it is necessary to delve into these differences by applying moderated T-tests with FDR correction in order to identify the metabolites responsible for these observations. In Fig. 4, Venn diagrams with the results obtained in the T-tests are shown. Fig. 4A shows the condition results (apple, pulp, leaf and peel) compared to basal. Different entities were differentially produced by leaf treatment (193), by apple treatment (259), by peel treatment (289), and by pulp treatment (254). Regarding to LPS treatment, Venn diagrams revealed significant differences between the evaluated extracts and LPS-treated cells. This was consistent with the results obtained in the hierarchical clustering, where extracts and LPS were grouped in different clusters.

According to Venn diagrams (Fig. 4B), it was observed that Leaf_L, Apple_L, Peel_L and Pulp_L presented different entities and therefore, a particular metabolic effect could be suggested. This observation was also reported above in cells treated only with extracts (Fig. 4) and it agrees with the results obtained in the hierarchical clustering analysis (Fig. 3B).

In Fig. 5 Venn diagrams of LPS, apple extracts (Leaf, Peel, and Pulp) as well as the apple extracts with LPS (Leaf_L, Peel_L and Pulp_L) are shown. The results revealed that the percentage of similarity in terms of the number of entities significantly altered between extract-treated cells and LPS-treated cells was lower than 20 %. Nevertheless, the percentage of similarity between extract + LPS-treated cells and LPS-treated cells, it is not only <20 % (except for leaf), but it is also practically the same as that reported by the extract treatment. Moreover, by comparing the similarity index between cells treated with an extract under a pro-inflammatory environment and in normal conditions, this proved to be>50 % in all cases.

3.4. Metabolic pathways

3.4.1. Amino acid metabolism

Several pathways belonging to amino acid metabolism have shown, to a greater or lesser extent, alterations in treated cells compared to untreated cells: aminoacyl-tRNA biosynthesis; valine, leucine and isoleucine biosynthesis; alanine, aspartate and glutamate metabolism; phenylalanine metabolism; arginine biosynthesis; arginine and proline metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; and glycine, serine and threonine metabolism (Fig. 6). Supplementary Table 1 shows the statistically altered metabolites in amino acid metabolism by LPS, extracts and extract + LPS treatments compared to untreated cells (Basal). β-alanine, alanine, glycine, glutamate, isoleucine, leucine, phenylalanine, proline, threonine, and valine were found differentially produced by treated cells. An increase in amino acid biosynthesis was observed by all conditions, except in the case of peel and LPS co-treatment. A down-regulation pattern for β-alanine and glycine was observed.

Amino acid metabolism plays an important role in many cellular processes not only for protein synthesis, but also for de novo synthesis of branched-chain fatty acids (BCAAs), as well as for the synthesis of purines and pyrimidines. Furthermore, amino acids are known to be involved in the regulation of the immune response by activation of lymphocytes, macrophages and natural killer cells; modulation of cellular redox state; gene expression; lymphocyte proliferation; and the production of antibodies, cytokines and other cytotoxic substances (P. Li, Yin, Li, Woo Kim, & Wu, 2007).

The metabolism of aminoacyl-tRNA biosynthesis as well as valine, leucine and isoleucine biosynthesis, were reported to be significantly altered in all the evaluated conditions, except in Peel_L. It is known that BCAAs, referring to leucine, isoleucine, and valine, can act as donors of

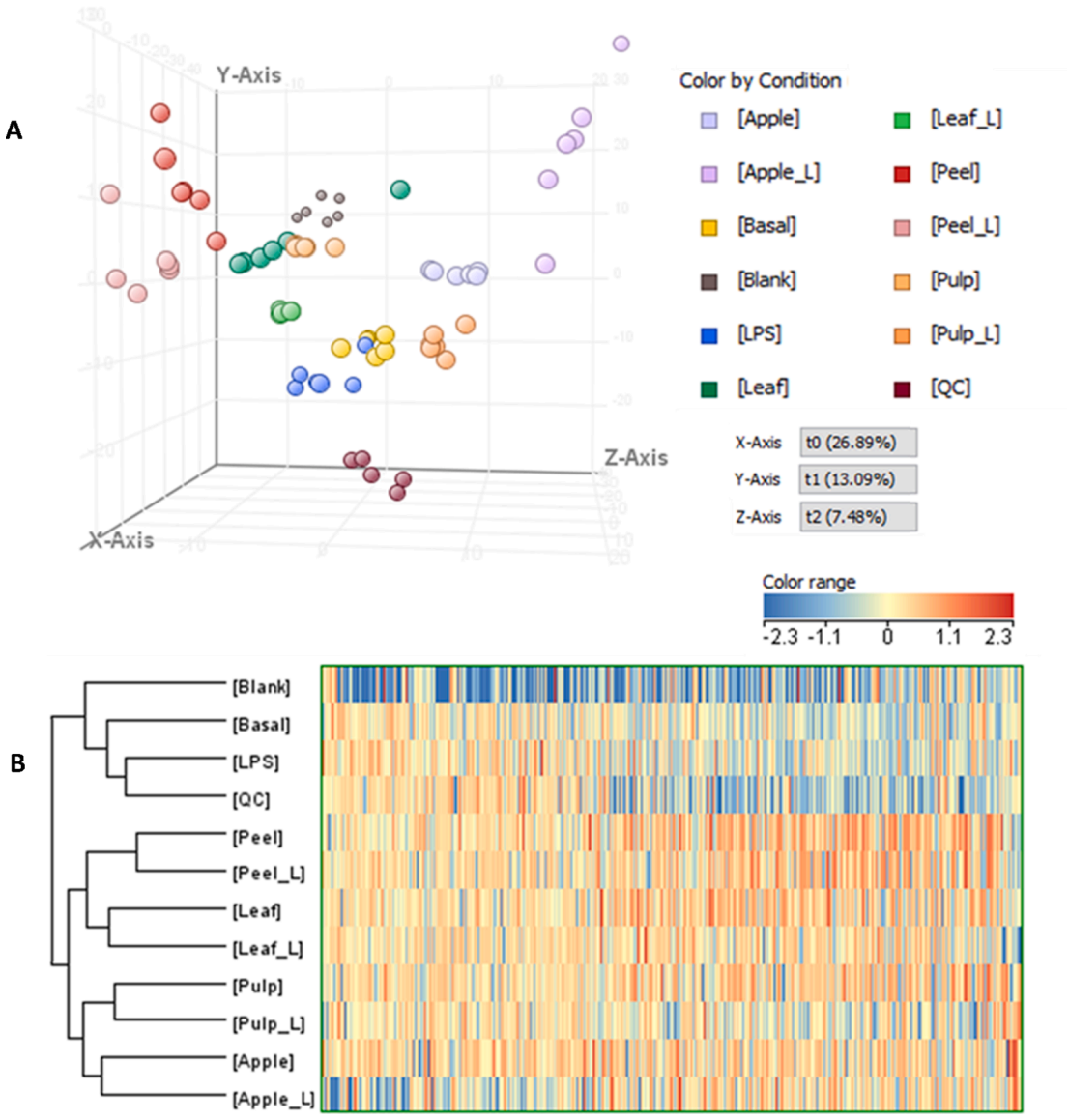

Fig. 3. (A) PLS-DA score plot (accuracy of the model of 90.538 %, R2 = 0.348 and Q2 = 0.031). (B) Hierarchical clustering analysis by applying Pearson's uncentered similarity measure and complete linkage.

nitrogen and of carbon skeletons for the synthesis of other amino acids that are important in supporting immune cell function (De Simone et al., 2013). Moreover, leucine is an activator of the mTOR signaling pathway that regulates protein synthesis and degradation in cells (Meijer & Dubbelhuis, 2004).

Alanine, aspartate and glutamate metabolism was also found significantly altered in apple extracts assay. Another intermediary, glutamate, was found up-regulated by almost all conditions, which could feed into the tricarboxylic acid (TCA) cycle to be converted to α-ketoglutarate, by glutamate dehydrogenase, connecting TCA with Glutamine/Glutamate cycle. TCA cycle is a key metabolic point located in the mitochondria that coordinates the metabolism of carbohydrates, proteins, and fats into carbon dioxide and adenosine triphosphate (ATP) (NOE & Mitchell, 2019). In most cells, the TCA cycle is essential for the maintenance of cell bioenergetics requirements, since produces reduced nicotinamide adenine dinucleotide (NADH) that fuels the electron transport chain (ETC). This also governs mitochondrial oxidative phosphorylation system (OXPHOS) that is responsible for ATP production. In addition, the TCA cycle can serve as a biosynthetic pathway by producing intermediate metabolites for de novo production of glucose, fatty acids, and nonessential amino acids (NOE & Mitchell, 2019). Furthermore, it was reported that TCA metabolites are important in controlling monocyte/macrophage phenotypes, and effector functions (NOE & Mitchell, 2019). The dual role played by macrophages in initiation and resolution of inflammation requires cells to adopt different processes. Therefore, macrophages must be able to switch rapidly from a resting to an activated state, leading to changes in their metabolism, some of which are hallmarks for a specific phenotype (Williams & O'Neill, 2018). Pro-inflammatory macrophages up regulate glycolysis and the pentose phosphate pathway (PPP) while the TCA is broken at two points and the fatty acid oxidation (FAO) and OXPHOS are down-regulated (Galván-Peña & O'Neill, 2014). Murine anti-inflammatory macrophages also up regulate glycolysis, whilst the TCA cycle remains intact and OXPHOS is functioning normally downregulated (Galván-Peña & O'Neill, 2014). In our trials, two intermediaries of the TCA cycle (citrate and fumarate) were found up regulated under apple extracts treatments in addition to LPS treatment. Citrate was reported to support both pro- and anti-inflammatory macrophage functions through various mechanisms: Under pro-inflammatory stimuli, citrate conversion to acetyl-coenzyme A (acetyl-CoA) results in phospholipid biosynthesis

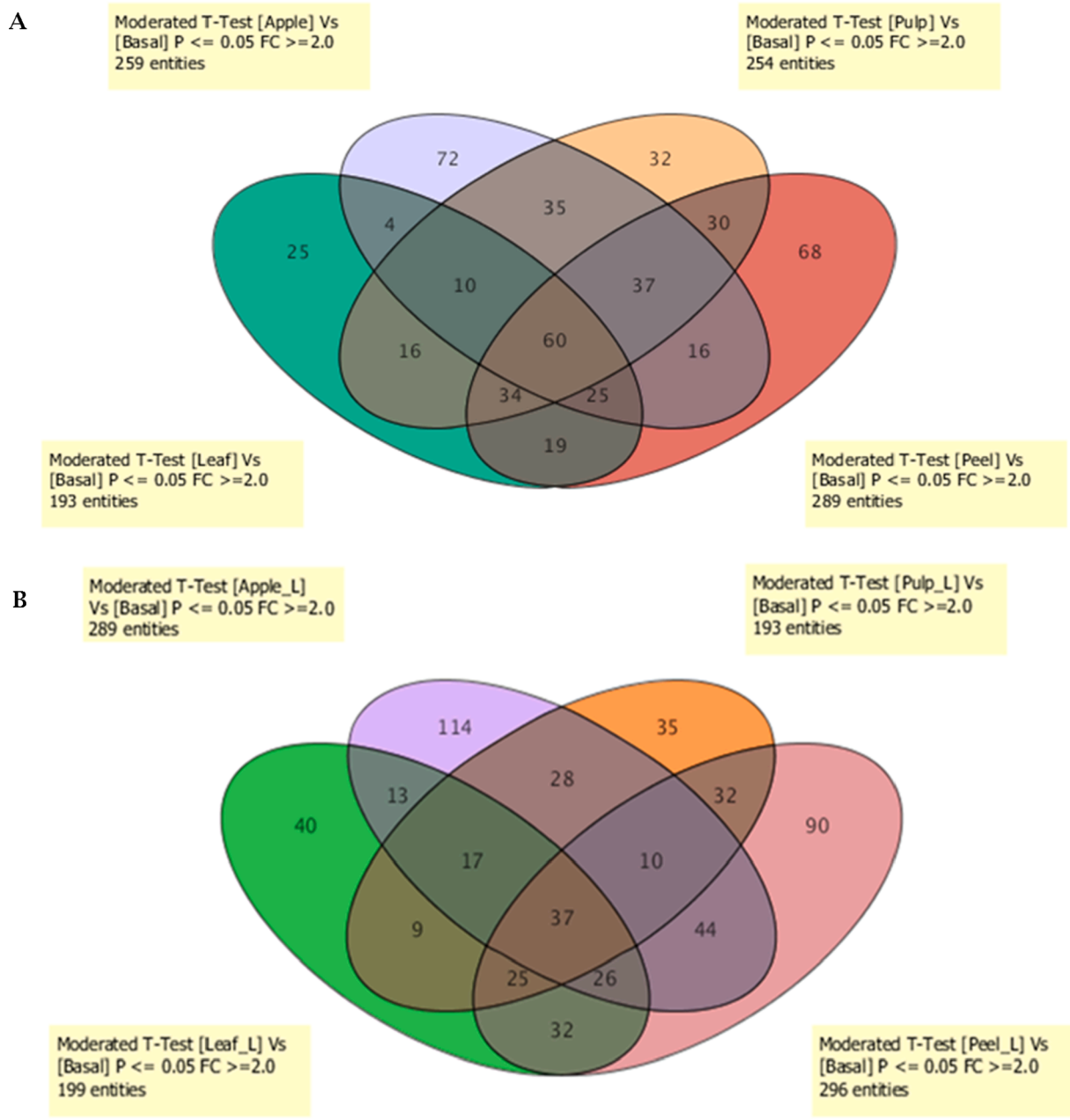

Fig. 4. Venn diagrams of the subsequent evaluated conditions: (A) apple, pulp, leaf and peel compared to basal; and (B) apple_L, pulp_L; leaf_L and peel_L compared to basal.

and prostaglandin E2 (PGE2) production and contributes to NADPH-dependent inducible nitric oxide synthase (iNOS) activity resulting in increased NO production. Moreover, under anti-inflammatory stimuli, increased availability of nuclear acetyl-CoA and subsequent histone acetylation, that regulates specific patterns of gene expression. It also was reported to inhibit glycolysis which is necessary for driving maximal pro-inflammatory macrophage responses (NOE & Mitchell, 2019). On the other hand, fumarate appears to serve as a feed-back mechanism to attenuate excessive inflammation by activating anti-oxidant and anti-inflammatory responses.

3.4.2. Carbohydrate metabolism

Results evidenced differences in carbohydrate metabolism, specifically in two pathways: galactose as well as fructose and mannose metabolism (Fig. 6). Supplementary Table 1 shows the noticeable difference in terms of the reduction in the production of glucose and mannose, respectively, in those cells exposed to leaf (1.04- and 1.002-fold decrease), apple (8.9- and 5.9-fold decrease), and pulp extracts (1.5- and 13.6-fold decrease); with no observable changes in cells exposed to peel or LPS. However, the response in those cells co-treated with LPS and apple and leaf extracts, showed increased production of both monosaccharides; in the same way that peel + LPS co-treated cells showed an increase in glucose production, while pulp + LPS co-treated cells did not present alterations in these compounds. Other entities related with carbohydrate metabolism that were found dysregulated were dihydroxyacetone phosphate, up-regulated by all conditions except by pulp which showed a down-regulation pattern, and peel_L that was unaltered; and two polyols: inositol, down-regulated by LPS and extracts, but unaltered in co-treated cells; and sorbitol, that was found mainly up-regulated by all conditions.

Although glycolysis has not been reported as a significant pathway in our trials, two intermediaries were found significantly dysregulated

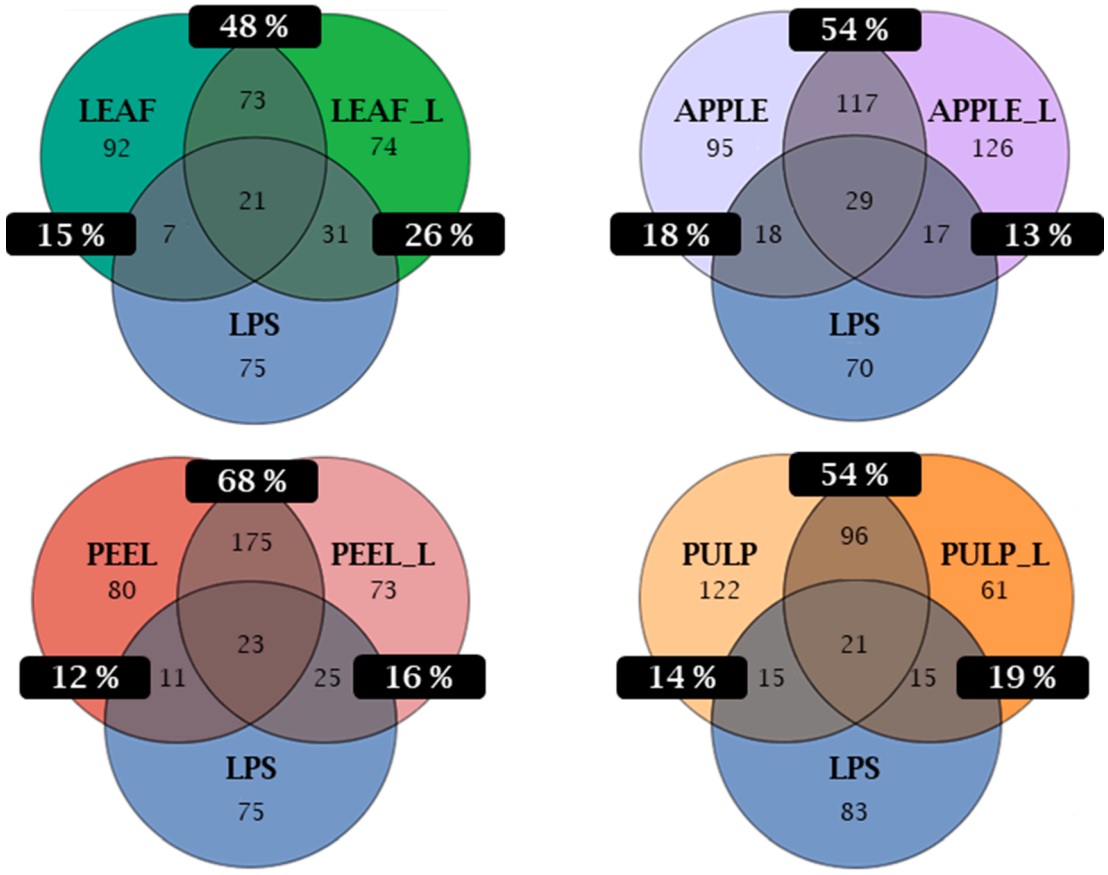

Fig. 5. Venn diagrams of the evaluated conditions: LPS, Lipopolysaccharide; Leaf; Apple; Peel; Pulp; Leaf_L, Leaf + LPS; Apple_L, Apple + LPS; Peel_L, Peel + LPS; and Pulp_L, Pulp + LPS; compared to basal. Percentages indicate the degree of similarity in terms of shared entities among the compared conditions, expressed in %.

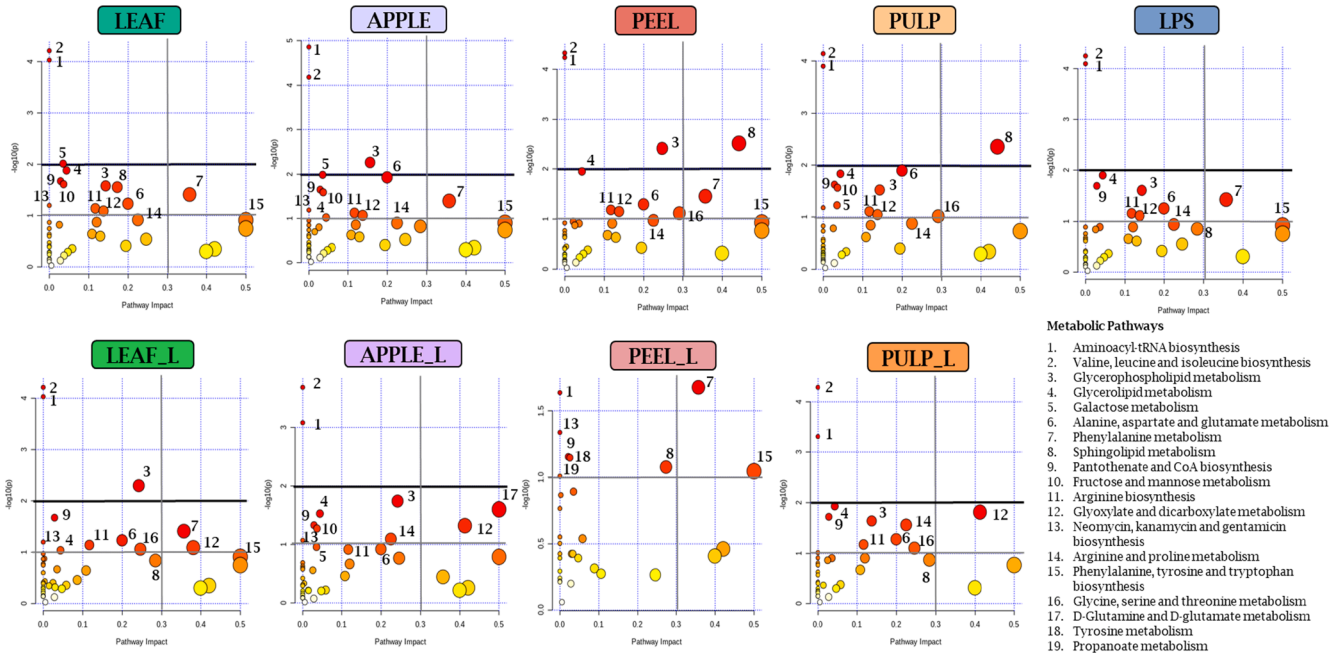

Fig. 6. Metabolome overviews of the evaluated conditions: LPS, Lipopolysaccharide; Leaf; Apple; Peel; Pulp; Leaf_L, Leaf + LPS; Apple_L, Apple + LPS; Peel_L, Peel + LPS; and Pulp_L, Pulp + LPS; compared to Basal. The top-pathways are ranked by the gamma-adjusted p values for permutation per pathway (y-axis) and the total number of hits per pathway (x-axis). The colour graduated from white to yellow, orange and red, circle size (large > small) as well as the values of both × and y increase represents the degree of significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(glucose and DHAP) in apple extracts besides other pathways related to carbohydrate metabolism: Galactose, fructose and mannose metabolism. However, it is noteworthy that glucose and mannose levels have been down regulated by leaf, apple and pulp treatments; while LPS treatment did not report changes for these metabolites. This increase in glucose consumption can occur in order to supply the bioenergetic requirements of an activation state of macrophages, since it leads to ATP production and provides metabolic intermediates for biosynthetic pathways of ribose, amino acids, and fatty acids that are essential for metabolic adaptation of the cell (Viola, Munari, Sánchez-Rodríguez, Scolaro, & Castegna, 2019). Although the behavior of LPS-treated cells does not agree with that previously reported in the literature, the metabolomics study of Abuawad et al. (2020) also did not report alterations in glycolytic intermediates in THP-1 derived macrophages treated with LPS. This highlights the heterogeneity and phenotypic diversity of macrophages under different stimuli. In such a way that the type of stimulus as well as culture conditions and exposure times could influence the response of this cell type (Abuawad et al., 2020).

3.4.3. Lipid metabolism

The most significant differences were found in lipid metabolism in the subsequent three pathways: glycerophospholipid metabolism, glycerolipid metabolism, and sphingolipid metabolism (Fig. 6).

Among glycerophospholipids, metabolic alterations were found in lysophosphatidylethanolamine (LysoPE), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI), dihydroxyacetone phosphate (DHAP), ethanolamine, glycerol-1-phosphate and *O*-phosphoethanolamine entities (Supplementary Table 1).

LysoPE exhibited a differential pattern between extracts (up-regulated) and LPS (down-regulated), showing peel and pulp the highest biosynthetic activity in these compounds. In the case of co-treated cells (extract + LPS), only minor upward alterations were observed in apple_L and peel_L conditions. Regarding PE, minor changes were observed by LPS treatment where only two unsaturated entities showed up-regulation [PE(22:1(11Z)/15:0) and PE(22:4(7Z,10Z,13Z,16Z)717:0)]. The treatment with extracts and the co-treatment of extract and LPS, differences were observed between both situations. Extract treatments mainly showed an up-regulation pattern in the production of saturated PE while co-treated cells showed a greater alteration in unsaturated representatives, mainly up-regulated except for pulp_L treatment, where down-regulation prevailed. In PG, the difference is even more remarkable, where up-regulation predominates in extract treatments (although these changes are minimal in leaf treatment); whilst LPS treatment showed few down-regulated entities and the co-treated cells remained unchanged with respect to these compounds. Dysregulations in PI were minimal, since only peel treatment showed upregulation of PI(P-20:0/17:0). Finally, PS alterations were reported mainly in co-treated cells that showed to a greater extent an upregulated pattern, while only LPS and pulp treatments showed up-regulation of PS (22:1(11Z)/17:0) and down-regulated production of PS(P-20:0/16:0) by LPS.

Regarding to glycerolipid metabolism, three intermediaries were found significantly altered in THP-1 macrophages under the influence of all tested conditions except for peel_L treatment, as is reflected in Supplementary Table 1: dihydroxyacetone phosphate (DHAP), glycerol 1-phosphate (G1P) and 1,3-dihydroxyacetone (DHA).

Alterations in sphingolipid metabolism were observed in terms of ceramides, lactosylceramides, C16 sphinganine, *O*-phosphoethanolamine and phytosphingosine production (Supplementary Table 1). Ceramides were found both upregulated and downregulated in LPS-stimulated THP-1 macrophages; apple extracts treatment exhibited a very significant upregulation pattern except by leaf treatment that showed no changes with respect to untreated cells; and in the case of co-treatment, significant modulation of ceramides was reported both up and downregulated. Regarding lactosylceramides, alterations in their production were only found in co-treated cells, with both up and down regulation pattern. C16 Sphinganine was found upregulated in leaf, peel and pulp treated cells but no changes were reported for the rest of conditions. The same pattern was observed for phytosphingosine. Finally, in the case of *O*-phosphoethanolamine, a significant enhancement was detected after LPS, leaf, apple, pulp and apple_L treatments, whilst peel showed a decrease in that compound, and peel_L and pulp_L remained unaltered.

Generally, lipid metabolism contributes to the functions of macrophages by meeting energetic requirements and modulating membrane fluidity (Mukundan et al., 2009). Glycerophospholipids (GLs) are components of cell membrane and lipoproteins which are involved in multiple biological processes, such as inflammation and cell differentiation (Zhang et al., 2017). A general enhancement in the composition of glycerophospholipids was observed in treated cells compared to untreated cells, among which cells treated with the extracts reported the most significant increase. This results agrees with recent studies that reported an increase in the composition of several glycerophospholipid species by using polarised THP-1 cells into pro- and anti-inflammatory phenotypes (Abuawad et al., 2020; Zhang et al., 2017). Regarding to glycerolipid metabolism, DHA, DHAP, G1P and glycerate connect glycerol catabolism with the glycolysis pathway and glycerophospholipids metabolism to fulfil its requirements. The regulation of glycerolipid biosynthesis is critical for homeostasis of cellular lipid stores and membranes (Zhang & Reue, 2017). Sphingolipids are one of the major lipid components of eukaryotic plasma membrane and are involved in several physiological functions, such as cell adhesion, signalling, immunity, skin barrier formation, neural functions, and glucose metabolism (Kihara, 2014). It is known that polarization of human macrophages was associated with differential regulation of sphingolipid mediators, sphingosine, and ceramide kinases (Martinez, Gordon, Locati, & Mantovani, 2006). Ceramides were found the main dysregulated entities, showing the co-treated cells the greatest alterations (both increased and decreased). Ceramides tend to self-associate in the cell membrane forming microdomains or platforms, traducing signalling cascades in immune cells. In macrophages, increased de novo ceramide biosynthesis is required for autophagosome formation, which is thought to have key roles in innate immunity (Maceyka & Spiegel, 2014). Therefore, ceramides metabolism increasement could be explained due to possible greater activity in signal transduction of co-treated cells, promoted by the double stimulation.

3.4.4. Metabolism of vitamins and cofactors

Pantothenate and CoA biosynthesis was significantly altered in treated THP-1 derived macrophages (Fig. 6). As is reflected in Supplementary Table 1, among the intermediaries involved in this pathway were found up-regulated by all conditions except peel_L: β-alanine, L-valine and pantothenate (vitamin B5).

The overall enhancement of intermediaries of Pantothenate CoA biosynthesis (β-alanine, L-valine and pantothenate) could be explained due to the pivotal role of pantothenate in metabolism of carbohydrates, fats, and protein for energy production as well as in the production of phospholipids, amino acids, and fatty acids, among others (Gammoh., 2019). Pantothenic acid (Vitamin B5) is an essential nutrient for the synthesis of CoA that it is also involved in proteins, carbohydrates, and fats metabolism. CoA is the principal acyl carrier and is required for many synthetic and degradative reactions in intermediary metabolism, and is an essential cofactor in all living systems (Leonardi & Jackowski, 2007). Nevertheless, insufficient data were obtained to elucidate the implications of these observed changes in macrophage metabolism.

4. Conclusions

This study revealed evidence that apple pulp exerts inhibitory activity against the production of IL-1β in human THP-1-derived macrophages treated with LPS, without exhibiting cytotoxicity.

This study also provides an untargeted multiplatform metabolomics

approach (LC/GC–MS) to understand metabolic effects promoted by four apple extracts (leaf, apple, peel and pulp) under normal and inflammatory conditions, in human THP-1 derived macrophages.

All the evaluated extracts promoted alterations in the metabolism of amino acids, specifically in alanine, aspartate and glutamate metabolism. Nevertheless, only apple extracts were able to induce metabolic alterations in THP-1 derived macrophages related to lipid metabolism (mainly glycerophospholipid, glycerolipid and sphingolipid metabolism). Leaf and apple extracts induced alterations in carbohydrate metabolism with alterations in galactose metabolism.

Moreover, under co-stimulation with LPS and apple extracts, cells mimic the metabolic response of extract-treated cells. Therefore, apple extracts seem to counteract the molecular mechanisms triggered by LPS that leads to a pro-inflammatory phenotype in macrophages. Nevertheless, there are still many unanswered questions about the mechanisms of action triggered in THP-1 derived macrophages by the influence of the conditions evaluated in the present study. Not only with regard to the evaluation of apple and its constituents, but also in the study of polarized THP-1 macrophages due to its heterogeneity.

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CRediT authorship contribution statement

Noelia Cambeiro-Pérez: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Visualization. **María Figueiredo-González:** Methodology, Investigation. **María Rosa Pérez-Gregorio:** Conceptualization, Writing – review & editing, Supervision, Project administration. **Catarina Bessa-Pereira:** Methodology, Investigation. **Víctor De Freitas:** Funding acquisition. **Borja Sánchez:** Conceptualization. **Elena Martínez-Carballo:** Methodology, Conceptualization, Writing – review & editing, Supervision, Project administration.

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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111037.

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