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Anti-Platelet Properties of Apple Must/Skin Yeasts and of Their Fermented Apple Cider Products

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Abstract: Alcoholic beverages like apple cider are considered functional beverages with several health benefits, when consumed in moderation, which are mainly attributed to their microbiota and the plethora of their bioactive compounds. Among them, bio-functional polar lipids (PL) have recently been found in apple cider, which despite low quantities, have exhibited strong anti-inflammatory and anti-platelet properties, while fermentation seems to affect the functionality of apple cider's PL bioactives. The aim of the present study was to elaborate yeast strains isolated from the complex mixtures of apple surface and must yeasts for evaluating their effects on the anti-platelet functional properties of PL bioactives from their final fermented apple cider products. First, bio-functional PL were extracted and separated from the biomass of the different isolated apple surface/must yeast strains, and were further assessed for their anti-platelet potency against human platelet aggregation induced by the potent inflammatory and thrombotic mediator platelet-activating factor (PAF), or by a classic platelet agonist like adenosine diphopshate (ADP). Novel functional apple ciders were then produced from the fermentation of apple juice by elaborating the most bioactive and resilient yeast strains isolated from the apple must with optimum fermentation properties. PL bioactives extracted from these novel apple cider products were also further assessed for their anti-platelet properties against both the PAF and ADP pathways of human platelet aggregation. These novel cider products were found to contain PL bioactives with lower IC50 values (~40 μg) and thus increased anti-platelet potency against platelet aggregation induced by PAF and ADP. GC-MS analysis of the PL bioactives extracted from these novel apple ciders showed that apple cider PL bioactives are rich in monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), such as the omega-6 linoleic acid (LA) and the omega-3 alpha linolenic acid (ALA), with favorably lower levels for their omega-6/omega-3 PUFA ratio, which further support the observed strong anti-platelet properties putative anti-inflammatory potency for the apple cider PL bioactives. However, further studies are needed in order to elucidate and fully characterize the apple yeast strains that can be utilized for increasing the anti-inflammatory, anti-platelet and cardioprotective functional properties of their fermented apple cider products.

Keywords: apple cider; fermentation; yeast; anti-inflammatory; anti-platelet; bioactives; polar lipids; platelet activating factor; MUFA; PUFA

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1. Introduction

Fermented alcoholic beverages, including apple cider, have been shown to provide several functional properties with health benefits when consumed in moderation. The beneficial functional properties of apple cider have been attributed to the plethora of its bio-functional compounds, including its phenolic content and anti-inflammatory and

anti-platelet polar lipids (PL) [1,2]. More specifically, fermentation of apple juices from different apple varieties produced apple ciders containing PL and phenolic bioactives with anti-inflammatory and anti-platelet properties against human platelet aggregation induced by the inflammatory and thrombotic mediator, platelet activating factor (PAF), but also by a classic platelet agonist, adenosine diphosphate (ADP) [1]. Similar outcomes were also observed in the wastes, apple cider by-products (apple pomace), from all these apple cider production procedures [2].

In addition, PL bioactives with similar anti-inflammatory potency have also been found in several yeasts/bacteria strains utilized for the production through fermentation of other alcoholic beverages like wine and beer, but also of bioethanol production, while both beer and wine products from such fermentations were also found to possess PL and phenolic compounds with strong antithrombotic and cardioprotective properties [3–5]. In all these alcoholic beverages including apple cider, it was reported that fermentation process affects the anti-inflammatory and anti-platelet functional properties of the final fermented product [1,3–5].

Even though apple cider can be simply defined as fermented apple juice, yet the process and specifications can be much more complex than its definition. Several parameters affect cider production, with yeast strains and yeast-available nutrients being crucial factors for the functional properties, nutritional value, and taste characteristics of apple cider [6–11]. There are specific yeast species that are associated with cider fermentation. In countries such as Ireland, Spain, and France, cider is mainly produced from naturally developing yeast species, derived from the fruit or sometimes from the surface of the processing equipment, stimulating alcoholic fermentation [9–11]. The most prevalent yeast specie in alcoholic fermentation has been shown to be the indigenous *S. cerevisiae*, which is also known as a budding yeast for its ability to bud off the parent cell. It is used in cider, beer, and wine making and is used as a model eukaryotic cell in the fields of molecular and cellular biology.

Nevertheless, apart from *S. cerevisiae*, several other yeast species, including *Debaryomyces, Candida, Hanseniaspora/Kloeckera, Metschnikowia, Pichia/Hansenula, Schizosaccharomyces, Torulaspora, Williopsis*, and *Zygosaccharomyces*, have also been found to supplement *S. cerevisiae* and thus enhance flavor complexity and aroma intensity [9–11]. Subsequently, these yeast species can be used in wine and cider fermentation to improve the complexity of the wine and cider flavor. Although most of these strains cannot perform complete alcoholic fermentation individually, a mixture of these strains together with *S. cerevisiae* delivers a desirable method for alcoholic fermentation using the correct inoculation methods [9–11]. However, further research is needed on evaluating how each one of these different apple yeast species of cider fermentation affect the bio-functional properties of the final fermented apple cider products.

Thus, the purpose of this study was the isolation of different types of yeast species present in apple surface and apple musts from different apple varieties, depending of their tannin content, such as Jonagold, Dabinett, and Aston Bitter varieties with low, medium, and high in tannins content, respectively, in order to investigate their suitability for apple cider fermentations for producing fermented apple cider products with increased anti-inflammatory and anti-platelet functional properties.

For achieving such a goal, several yeast strains from the apple surface and apple musts of these three different apple varieties were isolated, while the yeast strains containing the strongest PL bioactives, were further utilized for producing different apple cider products by fermenting a commercially purchased controlled apple juice. The PL bioactives of the apple ciders produced by these fermentations were further studied for their potential anti-platelet properties against human platelet aggregation induced by either PAF or ADP, while structural elucidation and evaluation of their fatty acid profile was performed by GC-MS analysis, in order to evaluate structure activity relationships.

The first reported outcomes of the present study may facilitate the choosing of the most optimum and resilient yeast strains and apple varieties with a final goal to improve

the functional properties of apple cider, in terms of its anti-platelet, anti-inflammatory and cardioprotective potency.

2. Materials and Methods

2.1. Isolation of Yeasts Strains from Apple Surface and Musts

All glassware used in this experiment was autoclaved for 15 min at 121 °C to kill any microbes on the glassware in order to have sterile equipment. Wallerstein Nutrient Agar (WLN; Sigma-Aldrich, Dublin, Ireland), De man, Rogosa and Sharpe Agar (MRS Agar, Oxoid, Hampshire, UK), Nutrient Agar (NA; Oxoid), WLN agar supplemented with ethanol and WLN agar containing 100 ppm and 10 ppm cycloheximide were prepared with distilled water according to manufacturers' instructions. For this project, WLN was used for its presence of bromocresol green in the medium which *Saccharomyces spp.* yeast does not usually metabolize. This differentiation medium is used for mixed yeast samples to observe the alternating characteristics and properties. After the medium was autoclaved it was let cool to ~50 °C and then poured into sterile Petri dishes and left to set. Petri dishes were labeled, sealed, and stored in a refrigerator until needed. All preparation took place in a laminar flow to prevent any contamination of the plates.

Apples from three different varieties (Jonagold that has low tannin content, Dabinett that has intermediate tannin content, and Aston bitter that has high tannin content) were supplied by 'The Apple Farm' (Cahir, Co., Tipperary, Ireland). Using 20 cm^2 sterile templates used for bio burden control, two apples of each variety were swabbed using Copan environmental swabs (Copan, Brescia, Italy), within a laminar flow and aseptic conditions. Once the apples were swabbed, the resulting suspensions were then streaked onto the four different varieties of WLN, MRS, and NA and placed in a 25 °C incubator, as this is the optimum temperature for yeast and mold growth. They were left to grow for five days, after which the growth on each plate was recorded by carrying out plate counts. Colony identification followed the scheme of Morrissey et al. (2004) [9].

The musts from freshly squeezed apple juice that were tested were juices from these three different apple varieties of one day and fourteen days old, while all these were also tested again at twenty-one-day-old musts. Initially, a serial dilution technique was used, where 100 µL of sample was put into 900 µL of peptone water. One-hundred microliters of this sample was put into an Eppendorf tube containing another 900 µL of peptone water and so on, providing thus diluted samples of 1.0×10^{-1} up to 1.0×10^{-6} dillutions, done in duplicate for each must variety. The samples from Eppendorf tubes corresponding to dilutions of 1.0×10^{-4}, 10^{-5} and 10^{-6} were spread on plates using inoculation spreaders (Sarstedt AG & CO, Numbrecht, Germany) and incubated at 25 °C for five days.

The major yeast species in the apple surface and must that are involved in the fermentation process were *Saccharomyces cerevisiae*, *Hanseniaspora* spp., *Metschnikowia pulcherrima*, *Debaryomyces*, and *Brettanomyces* spp. identified based on their growth and appearance on the various WLN media, as well as their morphology under the microscope. The isolation and growth of the apple yeasts, for all the three types of apple varieties (Jonagold, Dabinett and Aston Bitter, respectively), in several mediums (i.e., pure WLN as well as WLN supplemented with ethanol or 10 ppm cycloheximide or even 100 ppm cycloheximide) was in good agreement with those reported by Morrissey et al. (2004) [11].

From these yeast species, 19 different strains were picked, nine being from the surface of the apples, labeled as 1 (of the *Saccharomycodes/Saccharomyces* specie); 2 (of the *Brettanomyces* specie); 4, 5i, 5ii, and 6 (of the *Metschnikowia* specie); and 7, 8, and 9 (of the *Hanseniasporaa* specie), respectively, but also 10 strains picked from the must, labeled as A (of the *Brettanomyces lambicus* specie), B (of the *Brettanomyces* specie), C (of the *Metschnikowia* specie), D (of the *Brettanomyces bruxellensis* specie), E (of the *Metschnikowia* specie), F (of the *Debaryomyces* specie), G (of the *Saccharomycodes/Saccharomyces* specie), H (of the *Debaryomyces* specie), and I and J (of the *Saccharomycodes/Saccharomyces* specie), respectively, which were isolated and cryopreserved.

The isolated yeast strains were frozen at −20 °C for cryopreservation, in order to be used in further analysis. A single colony of each isolate of interest was spread on WLN agar and allowed to outgrow. Then, a single 10 µL sterile loop head from each plate put into a tube of cryopreservation beads. In the tubes along with the beads there was a mix of nutrient agar (NA) and glycerol, which was used in order to reduce damaging of cell components from the formed ice crystals. For reviving the cryopreserved yeasts, these were grown up in liquid medium. Briefly, one bead from the tube was added to 5 mL of yeast peptide dextrose (YPD), which consisted of 10 g peptone, 5 g yeast extract, and 10 g dextrose per l. After overnight growth, this was then streaked on to WLN agar and incubated at 25 °C for five days and allowed to grow and multiply for further uses/analyses.

2.2. Flow Cytometric Identification and Profiling of Apple Microflora and Apple Cider Fermentation

The flow cytometer used in this study was an ACCURI C6 (Becton Dickinson, Basingstoke, UK). This instrument has four containers at the side which connect to it: (1) water, (2) waste, (3) cleaning fluid, and (4) Decontaminated fluid. When the instrument is turned on, it goes through a five-minute cleaning cycle, after which ethanol ran through for 10 min, followed by distilled water for 10 min. To prepare the suspensions for flow cytometry, 1 mL of the Ringer's solution that was present in the swabs was put into an Eppendorf tube. This was then centrifuged for five min at 8500 rpm. This leaves the cells separated from the fluid as a pellet. The supernatant was then removed and 94 µL of staining buffer (SB) was added, along with five µL of wheat germ agglutinin (WGA) and one µL of SYBR gold. The staining buffer (SB) used was a standard phosphate-buffered saline (PBS) solution which contained 0.1% bovine serum albumin (BSA) which had a pH of seven. This was incubated at 37 °C for 15 min and centrifuged for a second time for another five min. The supernatant was removed again and finally 3 µL of propidium Iodide (PI) and 297 µL of staining buffer were added. Each Eppendorf tube was then vortexed and placed on the flow cytometer platform, where the needle was suspended in the fluid, for performing the flow cytometric analysis.

The flow cytometric analysis implemented in the present study allowed a better understanding and insight into the processing procedures effect on the yeast during apple cider production through the use of staining [12–14]. The information of the cell cycle and physiology was acquired by recording the DNA composition of the cells and observing the cell membranes integrity [12]. A specific gating strategy was implemented for identifying the cells based on specific dyes for the flow cytometric analysis of apple surface (1–9) yeast strains, as previously described [12–15]. More specifically, in step 1 the identification of apoptotic cells with less DNA/Chromosomes was based on DNA fluorescence (SYBR Gold), and granularity (side scatter (SSC)), while the percentage of apoptotic cells and non-apoptotic cells was also measured, as a measure of strength of the culture. In step 2, cells were tested for their permeability to PI, with PI positive cells being the permeabilized ones, while the PI-negative cells being the intact ones. In step 3 both permeable and intact cells were assessed in SYBR Gold and Wheat Germ Agglutinin (WGA). WGA binds to the chitin in bud scars. Bud scars are located on the cell wall from which new cells have budded and have left a scar. Thus, cells staining highly positive for WGA are likely to be those with many bud scars, in other words, cells which have given rise to daughter cells [15]. In order to test if intact or permeable cells had more or less bud scars, the intact and permeabilized populations were gated and their percentage of WGA-positive cells determined. The more the permeability found in cells, the more damaged the culture. In step 4 enumeration was conducted in the WGA-positive and WGA-negative cells from each of the populations. Thus, such a flow cytometric approach facilitated the measurement of the percentage of WGA positive cells. Most of the permeabilised cells were WGA-positive, as the permeabilised cells would be older and have more bud scars than the non-permeabilised cells. Overall, yeast strains originated from the musts were less permeable and therefore less susceptible to damage or apoptosis/cell-death. The apple skin yeast species showed a significant amount of apoptosis in comparison to the must

yeast species (100 times more). Based on this outcome, further fermentations for producing apple ciders to be assessed for functional anti-platelet properties, yeast strains from the apple must were chosen.

2.3. Fermentations

Commercially purchased apple juice (Squeez), with 10 to 12 degrees Brix and 0.1 g of protein that yeasts need to start fermentation, was initially autoclaved at 121 °C for 15 min. Nineteen large-scale fermentations were carried out; one for each yeast strain (1–9 & A–J) and a control commercial yeast winemaking strain (*Saccharomyces cerevisiae* "Flor"). 98 mL of apple juice was added to two mL of the yeast suspension in a conical flask, giving a 1:50 dilution factor. The flasks were weighed after the addition of solution and weighed again one week later, and measurements were recorded. Fermentation caused loss of weight due to the conversion of sugar to CO_2 and ethanol and the loss of CO_2 to the atmosphere. The flasks were incubated at 25 °C for seven days and then tested using a densitometer for specific gravity, while the pH was also measured. Apple must strains showed much higher % efficiency of fermentation (approximately 70–95%) than the surface strains that showed lower efficiency (approximately 4–40%) and produced lower quality apple cider end-products, which still contained high amounts of sugar (and presumably very little yeast metabolites). Thus, large scale fermentations for producing functional apple cider products were based mainly to fermentations in which the apple must yeast strains were utilized, and especially the A, B, F, G, and I apple must yeast strains that were found to contain the most bioactive PL with the strongest anti-inflammatory potency.

2.4. Extraction and Separation of Lipid Bioactives from Apple Musts Yeast Strains and from Their Apple Cider Fermented Products

The total lipids (TL) from each apple yeast strain, but also from the produced by the large-scale fermentations apple ciders, by utilizing the most bioactive apple must yeast strains with the most optimum fermentation profiling, were further extracted based on the Bligh and Dyer extraction method [16], as previously described [1,2]. Briefly, the TL extraction was achieved by homogenization of the sample (yeast strain biomass/apple cider) in a monophasic system containing chloroform/methanol/water in a 1:2:0.8 ($v/v/v$) ratio. Addition of appropriate volumes of water and chloroform was then performed in order to adjust the chloroform/methanol/water-based homogenate at a ratio of 1/1/0.9 ($v/v/v$) to achieve phase separation with the TL being present in the lower phase. This phase was gathered in round-bottom flasks and evaporated until dry on a flash rotary evaporator at 37 °C under vacuum between 700 and 50 mbar (Buchi Rotavapor, Mason Technology Ltd., Dublin, Ireland), and then re-dissolved in a chloroform/methanol solution at a ratio of 1/1 (v/v) and transferred to a small glass pre-weighted tube, which was evaporated under nitrogen stream (BOC, Dublin, Ireland). The obtained TL were then weighted and stored under nitrogen environment at −20 °C for a maximum of 8 weeks.

The obtained TL extracts of all samples were then further separated into their PL and Neutral Lipids (NL) fractions, based on the counter-current distribution method of Galanos and Kapoulas [17], as previously described [1,2]. Briefly, in order to obtain both NL and PL extracts, pre-equilibrated petroleum ether and 87% ethanol to water solution were used, with completion of this method yielding the PL in the ethanol phase and the NL in the petroleum ether phase within a separatory funnel. Then, both phases were collected in round-bottom flasks and further evaporated using a rotary evaporator until dry, while similarly to the TL extracts they were re-dissolved in a chloroform/methanol solution at a ratio of 1/1 (v/v), transferred to a small glass pre-weighted tube, evaporated under nitrogen stream until dry, and weighted and stored under nitrogen environment at −20 °C for a maximum of 8 weeks until further analysis.

All extractions and separations of lipid bioactives from each sample were performed in triplicates (n = 3) in order to ensure reproducibility of results. All glass consumables and solvents of analytical grade used for these extractions and separation procedures were purchased from Fisher Scientific Ltd. (Dublin, Ireland).

2.5. Platelet Aggregometry Biological Assays

The evaluation of the anti-platelet properties of the PL extracts from all nineteen apple yeast strains, but also from the fermented apple ciders of the most bioactive apple must yeast strains, were performed in human platelet rich plasma (hPRP) preparations from healthy donors, as previously described [18–20], in order to assess their ability to inhibit aggregation of human platelets induced by the inflammatory and thrombotic mediator PAF and by the well-established platelet agonist ADP. All platelet aggregation bioassays were carried out in a Chronolog-490 two channel turbidimetric platelet aggregometer (Havertown, PA, USA) coupled to the accompanying AGGRO/LINK® software (Version Opti8 for performing Aggregation, CHRONOLOG, Havertown, PA, USA) package, analyses. For the sampling of blood, 20 G safety needles and evacuated sodium citrate S-monovettes were used, which were purchased from Sarstedt Ltd. (Wexford, Ireland), while the isolation of hPRP was performed through centrifications using an Eppendorf 5702R centrifuge (Eppendorf Ltd., Stevenage, UK), as previously described [18–20]. A Shimadzu UV-1800 spectrophotometer (Kyoto, Japan) was used with a quartz 1 cm cuvette for the spectrophotometric analysis. All the other materials, consumables and reagents for platelet aggregation were purchased from Labmedics LLP (Abingdon on Thames, UK) and Chronolog (Havertown, PA, USA), apart from standard PAF and BSA that were purchased from Sigma Aldrich (Wicklow, Ireland) and aspirin (Bayer, Berlin, Germany) that was purchased from a pharmacy (Bayer, Berlin, Germany).

The 50% inhibitory concentration value—IC50 value (half-maximal inhibitory concentration) for each sample was calculated by the amount (μg) of the lipid sample that led to 50% of inhibition of human platelet aggregation induced by PAF or ADP in hPRP platelet suspensions of 0.250 mL, as previously described [18–20]. Briefly, a range of concentrations for each lipid sample was assessed and a linear and dose-dependent relationship of the inhibitory effects of the PL bioactives with the concentrations of each lipid sample tested was observed, within the 20–80% range of the percentage of inhibition against PAF/ADP induced aggregation of hPRP. In order to derive such inhibitory curves, a range of 10 to 200 μg amount of lipids were assessed for the most bioactive yeast/apple cider PL, while a range of 50 to 500 μg was also utilized for the less bioactive ones.

From this derived curve for each lipid sample assessed, the concentration (μg) of the lipid sample that led to 50% of PAF/ADP induced aggregation of hPRP was calculated as the 50% inhibitory concentration value also known as the IC50 value (half-maximal inhibitory concentration) for each sample. Thus, the resulting IC50 values were expressed as a mean value of the mass of lipid (μg) in the aggregometer cuvette ± standard deviation (SD). Using blood samples from different donors, all experiments for evaluating the bioactivities of each lipid extract from each apple juice/cider sample were performed several times (n = 6), for each replicate, in order to ensure reproducibility.

2.6. Gas Chromatography–Mass Spectrometry (GC–MS)

The fatty acid (FA) profile of all PL samples was evaluated by GC–MS analyses of their fatty acid methyl esters (FAME) in a Varian 410-Gas Chromatographer coupled to a Varian 210-MS detector equipped with a split/splitless injector (Agilent Technologies, Palo Alto, CA, USA), as previously described [18,19]. The polar lipid bioactives assessed were obtained from the apple ciders, which were produced by the large-scale fermentations of the commercially purchased and autoclaved apple juice that was inoculated by the most bioactive apple must yeast strains showing the most optimum fermentation profiling, the standards and reagents used for GC-MS were supplied by Sigma Aldrich (Wicklow, Ireland).

Briefly, the FAME of apple cider PL bioactives were prepared using a solution 0.5 N KOH in 90% CH3OH and extracted with n-hexane. The FAME analysis was carried out using the internal standard method, by preparing a five-point calibration curve based on five solutions of heptadecanoic acid methyl esters (17:0—50 ppm, 100 ppm, 200 ppm, 400 ppm, 800 ppm) and heneicosanoic acid (21:0—five 500 ppm injections) methyl esters

standards. Five 1 μL injections of each solution were analyzed. The ratio of the mean is of heptadecanoic acid (17:0) to that of the internal standard (21:0) and is used as the *y*-axis variable, whereas the concentration (ppm) of 17:0 is used as the *x*-axis variable of the calibration curve. The equation that described the curve was: y = 0.0041x + 0.12 with an R^2 = 0.9969, where the ratio of the area of the analyte peak to that of the internal standard represents the y value for the above equation, subsequently the x-value represents the analyte concentration of a selected fatty acid in the lipid sample to be tested.

The separation of FAME was achieved on an Agilent J&W DB-23 fused silica capillary column (60 m, 0.25 mm, i.d., 0.25 μm; Agilent, Santa Clara, CA, USA). The injector was set at 230 °C with a split ratio of 1:5; the injection volume was 1 μL. The carrier gas was high purity helium with a liner flow rate of 1 mL/min. The oven temperature was initially programmed to 100 °C for 5 min, raised to 240 °C at 3 °C/min, and finally isothermal at 240 °C for 10 min. FAME were identified using a pre-derivatised 37-component FAME standards mix (Sigma Aldrich, Wicklow, Ireland) by comparison of the retention times and their subsequent obtained MS spectra of the relative peaks with the aid of the Varian Star Chromatography Workstation Version 6 software (Agilent Technologies, Palo Alto, CA, USA) and a NIST library (Gaithersburg, MD, USA).

2.7. Statistical Analysis

Normality for all IC50 values, lipid content and FA composition of the lipid sample studied, was tested using Kolmogorov–Smirnov criterion. Subsequently, one-way analysis of variance (ANOVA) was used for all comparisons of IC50 values against PAF and ADP, while comparisons in the lipid content and FA composition acquired from the GC–MS analysis were observed using the Kruskal–Wallis nonparametric multiple comparison test. Differences were considered to be statistically significant when the *p*-value was less than 0.05 ($p < 0.05$). The data were analyzed using a statistical software package (IBM-SPSS statistics 26 for Windows, SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Anti-Platelet Potency of PL Bioactives from the Apple Surface/Must Yeast Strains and from Their Fermented Apple Cider Products

Within the present study the most resilient, bioactive, and efficient for fermentations yeast strains of the indigenous yeast species *Saccharomyces cerevisiae*, *Hanseniaspora* spp., *Metschnikowia pulcherrima*, *Debaryomyces*, and *Brettanomyces* spp., isolated from apple must of three types of apple varieties depending on their tannin content (Jonagold, Dabinett and Aston Bitter, respectively), were further utilized for apple cider fermentations. For the first time, all these apples must strain, along with the apple ciders produced by the most bioactive and efficient for fermentations apple must strains, were also evaluated for the anti-platelet potency of their PL bioactives, while their fatty acid profile and structure activity relationships were elucidated by GC–MS-based lipidomics.

All PL extracts derived either from the apple surface/must yeast strains or from the apple ciders produced from fermentations of commercially purchased and autoclaved apple juice, by utilizing the most bioactive and efficient/resilient to fermentation yeast strains from apple must, were further assessed for their potential anti-platelet bioactivities against PAF/ADP-induced aggregation of human platelets. The results from these bioassays are expressed as IC50 values (half maximal inhibitory concentration), meaning the μg of PL extracts that can inhibit 50% of platelet aggregation induced by the PAF/ADP-pathway, as shown in Figure 1. Note that the lower the IC50 value for a lipid bioactive the stronger its inhibitory potency against PAF or ADP.

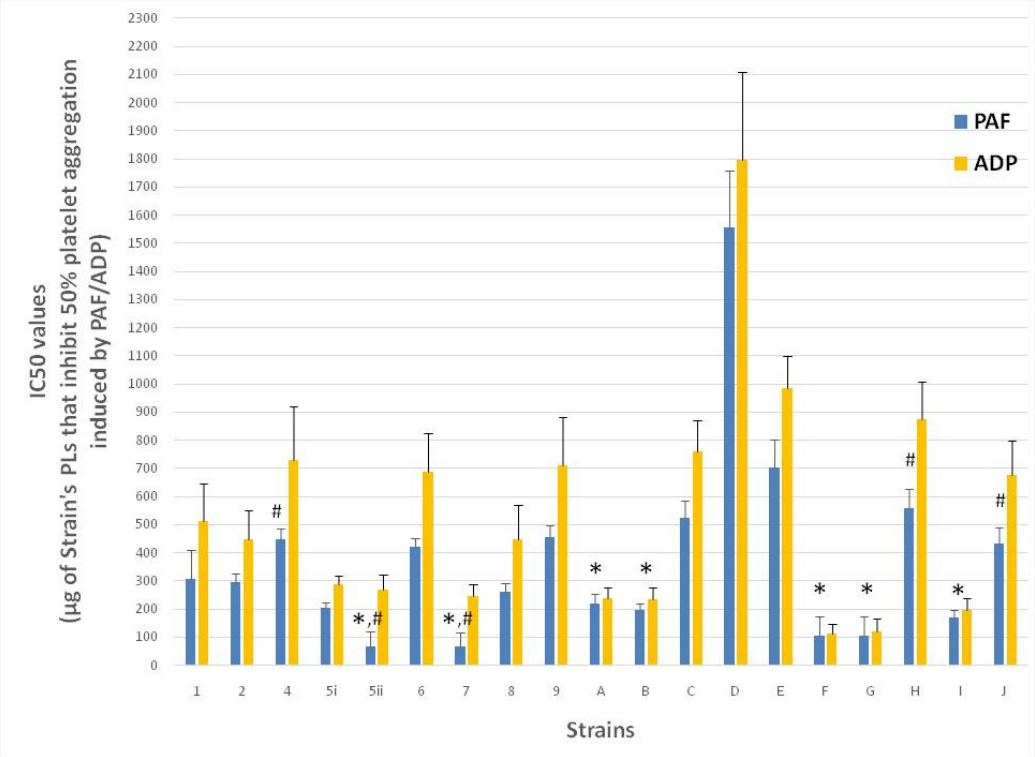

Figure 1. The anti-platelet potency of PL extracts from apple surface yeast strains (1, 2, 4, 5i, 5ii, 6–9) and apple must yeast strains (A–J), against human platelet aggregation induced by the inflammatory and thrombotic mediator PAF (blue bars) or by the platelet agonist ADP (yellow bars). Results are expressed as means of the IC50 (half-maximal inhibitory concentrations) values in μg of PL in the aggregometer cuvette that causes 50% inhibition of PAF/ADP-induced platelet aggregation (the lower the IC50 value for a lipid extract the higher its inhibitory effect against the specific agonist of platelet aggregation). * denotes statistically significant difference ($p < 0.05$) when the anti-PAF potency (IC50 value) of the bioactive PL extracts were compared within all strains assessed, while # denotes statistically significant difference ($p < 0.05$) when the anti-PAF potency (IC50 value) of the bioactive PL extracts from each strain was compared with the relative anti-ADP potency for this strain. Abbreviations: PL, polar lipids; PAF, platelet-activating factor; ADP, adenosine 5′ diphosphate.

Thus, there were differences observed in the bioactivities of PL extracts obtained from different yeast strains (Figure 1). More specifically, PL from 5i, 5ii, and 7 apple surface yeast strains and from the A, B, F, G, and I apple must yeast strains showed the strongest anti-platelet potency against the inflammatory PAF-pathway (low IC50 values against PAF-induced platelet aggregation). From these more bioactive yeast strains, only the A, B, F, G, and I yeast strains from apple must showed also strong anti-platelet potency against the ADP-pathway (low IC50 values against ADP-induced platelet aggregation), which were comparable to their anti-PAF bioactivities too (Figure 1). PL bioactives extracted from all the other yeast strains, namely, 1, 2, 4, 6, 8, 9, C, D, E, H, and J, respectively, exhibited low anti-platelet properties on human platelets, with the PL extracted from yeast strain D showing the lowest activities against both PAF and ADP.

The anti-platelet potency against the PAF and ADP pathways observed in the most bioactive PL from 5i, 5ii, and 7 apple surface yeast strains and from the A, B, F, G, and I apple must yeast strains, were found to be comparable with the anti-PAF and anti-ADP potency of other yeasts used in wine production [3] and bacteria used in bioethanol production [5], through fermentations. These outcomes further emphasize that microorganisms of biotechnological interest, which are mainly used for alcoholic fermentations, possess bioactive PL with anti-inflammatory and anti-platelet properties against the inflammatory and thrombotic pathways of PAF and ADP induced platelet aggregation. Thus, it is

possible that such bioactive PL found in several alcoholic beverages [3,4] and bioethanol fermentations [5], maybe originated from the yeasts/bacteria involved in the fermentation process. Nevertheless, further studies are needed to support such a notion.

Based on the above, the most bioactive and resilient/efficient for fermentations, apple must yeast strains A, B, F, G, and I were further utilized for producing their fermented apple cider (AC) products, namely, ACA, ACB, ACF, ACG, and ACI, respectively, by fermenting a commercially purchased and autoclaved apple juice, in order to have similar growth medium and conditions. The PL bioactives of these apple ciders were extracted and separated from their TL and NL, with Table 1 showing the yield of these extractions.

Table 1. The yield of extraction for the total lipids (TL), neutral lipids (NL) and polar lipids (PL) of the apple ciders ACA, ACB, ACG, ACG, and ACI, expressed as g/100 g of sample (mean ± SD, $n = 3$).

Samples	TL (g/100 g)	NL (g/100 g)	PL (g/100 g)
ACA	0.031 ± 0.021	0.006 ± 0.003	0.025 ± 0.011
ACB	0.036 ± 0.015	0.005 ± 0.002	0.031 ± 0.009
ACF	0.046 ± 0.022	0.004 ± 0.001	0.042 ± 0.005
ACG	0.051 ± 0.025	0.007 ± 0.002	0.044 ± 0.008
ACI	0.042 ± 0.009	0.006 ± 0.002	0.036 ± 0.011

Abbreviations: TL: Total lipids; NL: Neutral lipids; ACA, ACB, ACF, ACG, ACI: apple cider produced by apple juice fermentation with the strain A, B, F, G and I yeast strains.

The yield of TL, NL, and PL extract in these apple ciders was found to be of similar yield to previously reported yields of lipids extracted from apple ciders produced by fermenting apple juices of the three different apple varieties, inoculated by *S. cerevisiae* [1], but also with other beverages like wine, beer, and tea [3,4,21], but much lower from other food sources that are rich in lipids, such as marine-related food sources [18,19].

Of the PL and NL fractions retrieved from the TL extracts, there were significantly higher amounts present of PL, ranging from 80 to 95% of the TL, in comparison to NL ranging from 5 to 20% of the TL in all samples. Thus, the yield for PL approximately ranged from 0.015 to 0.075 g/100 g of the apple cider beverages. These results come also in accordance with previously reported ones for a higher yield in PL from apple ciders produced by fermenting apple juices of the three different apple varieties, inoculated by *S. cerevisiae* [1], but also with other beverages like wine, beer and tea [3,4,21].

The anti-platelet activities of PL extracts from these apple ciders against both PAF and ADP induced aggregation of human platelets, are shown in Figure 2. Results are again expressed as IC50 values, thus as μg of apple cider PL extracts that can inhibit 50% of platelet aggregation induced by the PAF/ADP pathway. Again, there were differences observed in the bioactivities of PL extracted from different apple cider product. More specifically, PL from ACF and ACG apple ciders showed the strongest anti-platelet potency against the inflammatory PAF-pathway and the most potent anti-platelet properties against the ADP-pathway too, as they exhibited the lowest IC50 values against both PAF and ADP-induced platelet aggregation (Figure 2).

PL from all the other apple ciders tested, namely, ACA, ACB, and ACI, exhibited lower, but still considerably strong anti-platelet properties against PAF and ADP on human platelets (Figure 2), which were found to be of similar potency when compared to the relative bioactivities that were previously observed in PL from apple ciders produced by fermenting apple juices of the three different apple varieties, inoculated by *S. cerevisiae* [1].

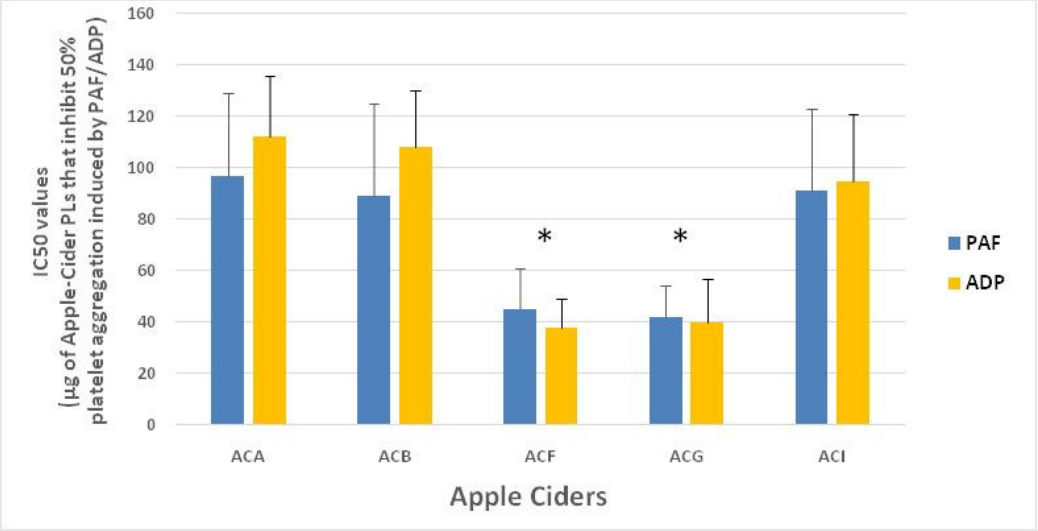

Figure 2. The anti-platelet potency of PL extracts from the novel functional apple ciders (AC) ACA, ACB, ACF, ACG and ACI, which were produced by fermenting apple juice inoculated with the A, B, F, G, and I apple must yeast strains, respectively, as assessed against human platelet aggregation induced by the inflammatory and thrombotic mediator PAF (blue bars) or by the platelet agonist ADP (yellow bars). Results are expressed as means of the IC50 (half-maximal inhibitory concentrations) values in μg of apple cider PL in the aggregometer cuvette that causes 50% inhibition of PAF/ADP-induced platelet aggregation (the lower the IC50 value for a lipid extract the higher its inhibitory effect against the specific agonist of platelet aggregation). * denotes statistically significant difference ($p < 0.05$) when the anti-PAF and/or anti-ADP potency (IC50 value) of the bioactive PL extracts were compared within all novel functional apple ciders. Abbreviations: PL, polar lipids; PAF, platelet-activating factor; ADP, adenosine 5′ diphosphate.

On the other hand, the strong anti-platelet properties of PL bioactives from apple ciders ACF and ACG were found to be two times stronger than the previously reported bioactivities for PL from the apple ciders produced by fermenting apple juices of the three different apple varieties, inoculated by *S. cerevisiae* [1], but were of similar potency to the bioactivities observed in PL from other beverages like wine, beer, and tea [3,4,21]. Thus, it seems that some PL bioactives of the apple cider products may be originated from their bioactive yeasts, either present in the cell membranes and co-extracted during the extractions of the apple cider products or being secreted exo-cellularly from the yeasts to the fermented apple must and thus to the final fermented cider product, as it was also been observed previously during wine and bioethanol fermentations [3,21], but also in marine microalga cultures [22].

Furthermore, in order to compare the anti-platelet potency of the most bioactive apple cider PL, a classic anti-platelet agent was also elaborated, the acetyl-salicylic acid (aspirin), which affects both the PAF and ADP pathways. It was found that in the conditions applied for the platelet aggregometry assay in this study, the acetyl-salicylic acid (aspirin) inhibited strongly both the PAF and ADP pathways of human platelet aggregation, with relative IC50 values of 15.2 ± 6.0 μg and 7.1 ± 5.4 μg, respectively, which were of similar potency with previously reported outcomes for aspirin against human platelet aggregation [21]. These outcomes further support the strong anti-platelet potency of the most bioactive PL bioactives found in apple ciders ACF and ACG, with comparable relative IC50 values of approximately 40 μg for the inhibition of both the PAF and ADP pathways, which even though lower from the observed anti-platelet effects of aspirin, still they had a considerably comparable anti-platelet effect that was within the same order of magnitude with that of aspirin (especially against PAF), and thus indicating putative cardioprotective preventative properties for these novel functional apple cider products.

In addition, taking into account that the IC50 value of 40 μg for the apple cider PL bioactives that were tested in 0.25 mL of PRP correspond to an approximate IC50 value of 0.16 mg/mL of plasma, while an average person contain approximately 2.5 L of plasma (the 55% of his/her blood volume), it seems that the presence of 400 mg of such PL bioactives (2500 mL × 0.16 mg/mL) in the plasma of a person may be able to provide some anti-platelet properties and preventative cardioprotective benefits. Moreover, note that according to the aforementioned yield of extraction for the PL from these apple ciders, the volume/amount of 0.5 L (a pint) of cider that is usually consumed contains approximately 200 mg of PL. Based on the above, the proposed moderate consumption of alcoholic beverages like apple cider (once/twice per week) within a well-balanced diet, it may be able to provide the appropriate amounts of PL bioactives with potent anti-platelet and cardio-protective properties. However, it should be stressed out that not all the amounts of PL that are consumed are then present in the plasma, as some of them are degraded/catabolized during digestion, while only a small proportion of some (5–20%) may remain intact during digestion, which however due to their amphiphilic properties such PL are easily diffused from the digestive tract to the blood stream where they preferably bind to specific plasma lipoproteins and cell membranes, affecting thus their bio-functionality. Nevertheless, more targeted dietary interventions based on moderate consumption of apple cider are needed in order to fully elucidate how much of the apple cider PL bioactives are present in the plasma in vivo that can provide anti-platelet, anti-inflammatory and cardioprotective properties.

Overall, these findings show that the apple ciders produced in this study contain PL bioactives with anti-platelet properties in agreement with the literature [1], which may reduce PAF induced thrombo-inflammation and ADP induced platelet activation and aggregation linked to several chronic disorders, such as cardiovascular disease (CVD), cancer, renal, and neurodegenerative disorders, persistent infections and their subsequent manifestations, etc. [23]. However, as mentioned above, more studies are needed, both in vitro in other disease cell models and in vivo in dietary interventions, in order to fully elucidate such an anti-platelet and anti-inflammatory potential for novel functional apple cider PL bioactives.

3.2. Fatty Acid Profile of PL Extracts Apple Ciders Produced from Apple Juice Fermented by Utilizing the Most Bioactive Strains of Apple Cider Must Microflora

Table 2 shows the fatty acid composition of the PL bioactives from apple ciders ACA, ACB, ACF, ACG, and ACI that were produced by fermenting a commercially purchased and autoclaved apple juice, by utilizing the most bioactive apple must yeast strains A, B, F, G, and I, respectively, which were also less permeable and less susceptible to cell damage/apoptosis, as obtained from the GC–MS analysis of these PL samples. All PL extracts from these apple ciders were found to be rich in saturated fatty acids (SFA) followed by lower amounts of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFAs), which were found to be of similar amounts (Table 2).

In all PL extracts of the apple ciders assessed, hexadecanoic acid (C16:0) was the most abundant SFA, while the most abundant MUFA was oleic acid (C18:1 c9), but in considerably lower amounts than the aforementioned SFA. In addition, these PL extracts were also rich in the essential omega-6 (n-6) PUFA, linoleic acid (LA) (C18:2 c9, 12 n-6), followed by the essential omega-3 (n-3) PUFA, alpha linolenic acid (ALA) (C18:3 c9,12,15 n-3), and by much less but considerable amounts of other bioactive n-3 PUFAs such as eicosapentaenoic acid (EPA) (C20:5 c5,8,11,14,17 n-3), docosapentaenoic acid (DPA) (C22:5 c7,10,13,16,19 n-3), and docosahexaenoic acid (DHA) (C22:6 c4,7,10,13,16,19 n-3). These results are in accordance with the previously reported fatty acid content of the bioactive PL extracts from apple products (apple juices from the apple varieties Jonagold, Dabinett and Aston Bitter and apple ciders produced by fermenting these apple juices by inoculating them with *S. cereviseae*) [1], from apple cider by-products of the same apple varieties [2], and of the PC and PE bioactives from the PL extract of these apple-based products/by-products [1,2].

Table 2. The fatty acid profile obtained of the PL of the apple ciders ACA, ACB, ACF, ACG, and ACI, expressed as the percentage of each fatty acid in the total fatty acids for each sample (mean ± SD, n = 3).

Fatty Acid	ACA	ACB	ACF	ACG	ACI
C13:0	0.43 ± 0.13	0.44 ± 0.09	0.23 ± 0.13	0.22 ± 0.11	0.43 ± 0.09
C14:0	4.73 ± 0.40	4.60 ± 0.20	4.26 ± 0.70	4.27 ± 0.72	4.40 ± 0.20
C15:0	0.91 ± 0.17	0.78 ± 0.18	0.78 ± 0.18	0.73 ± 0.10	0.77 ± 0.13
C16:0	29.56 ± 0.82	29.66 ± 0.85	29.50 ± 0.69	29.25 ± 0.79	29.42 ± 0.86
C16:1 c9	1.69 ± 0.11	1.69 ± 0.11	1.30 ± 0.21	1.33 ± 0.18	1.61 ± 0.14
C17:0	0.85 ± 0.12	0.85 ± 0.12	0.69 ± 0.17	0.37 ± 0.16	0.85 ± 0.08
C18:0	21.33 ± 0.67	22.17 ± 0.35	19.73 ± 0.70	19.13 ± 1.19	22.88 ± 0.69
C18:1 c9	16.73 ± 0.47	16.80 ± 0.92	17.08 ± 1.19	17.77 ± 0.55	16.67 ± 0.40
C18:2 c9,12 (LA)	12.30 ± 0.50	11.69 ± 0.38	10.79 ± 0.30	12.37 ± 0.24	11.36 ± 0.51
C18:3 c9,12,15 (ALA)	5.18 ± 0.41	5.15 ± 0.28	6.17 ± 0.48	6.69 ± 0.40	5.50 ± 0.40
C20:0	1.15 ± 0.05	1.19 ± 0.08	1.77 ± 0.15	1.11 ± 0.12	1.20 ± 0.10
C20:1 c11	0.98 ± 0.13	0.97 ± 0.15	1.37 ± 0.07	1.22 ± 0.13	0.96 ± 0.09
C20:3 c8,11,14	0.24 ± 0.07	0.22 ± 0.06	0.35 ± 0.07	0.21 ± 0.06	0.26 ± 0.05
C20:4 c5,8,11,14	0.37 ± 0.08	0.37 ± 0.08	0.57 ± 0.09	0.27 ± 0.05	0.35 ± 0.07
C20:5 c5,11,14,17 (EPA)	0.86 ± 0.09	0.83 ± 0.12	1.29 ± 0.15	1.09 ± 0.11	0.81 ± 0.12
C21:0	0.74 ± 0.15	0.74 ± 0.15	1.03 ± 0.24	0.74 ± 0.08	0.68 ± 0.11
C22:1 c13	0.90 ± 0.20	0.82 ± 0.15	1.51 ± 0.31	1.18 ± 0.18	0.80 ± 0.13
C22:5 c7,10,13,16,19 (DPA)	0.44 ± 0.11	0.41 ± 0.11	0.63 ± 0.20	0.84 ± 0.12	0.41 ± 0.12
C22:6 c4,7,10,13,16,19 (DHA)	0.63 ± 0.10	0.60 ± 0.14	0.94 ± 0.18	1.20 ± 0.10	0.62 ± 0.15
SFA	59.71 ± 0.61	60.43 ± 0.95	58.00 ± 1.64	55.82 ± 1.12	60.64 ± 0.27
MUFA	20.67 ± 0.08	20.28 ± 0.65	21.26 ± 1.29	21.50 ± 0.98	20.04 ± 0.29
PUFA	20.02 ± 0.53	19.29 ± 0.31	20.74 ± 0.47	22.68 ± 0.56	19.32 ± 0.27
n6	12.91 ± 0.44	12.30 ± 0.55	11.71 ± 0.18	12.85 ± 0.23	11.97 ± 0.46
n3	7.11 ± 0.29	6.99 ± 0.09	9.03 ± 0.51 *	9.83 ± 0.34 *	7.34 ± 0.42
n6/n3	1.82 ± 0.09	1.76 ± 0.07	1.30 ± 0.08 *	1.31 ± 0.02 *	1.64 ± 0.15

* indicates statistically significant difference ($p < 0.05$). Abbreviations: PL: polar lipids; ACA, ACB, ACF, ACG and ACI indicate apple ciders produced by fermenting a commercially purchased and autoclaved apple juice, by utilizing the most bioactive and less permeable and less susceptible to cell damage/apoptosis yeast strains A, B, F, G and I from apple must, respectively; c: cis; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; n3: omega-3 PUFA; n6: omega-6 PUFA; LA: linoleic acid; ALA: alpha linolenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid.

However, the finding that the PL bioactives from the apple ciders ACA, ACB, ACF, ACG, and ACI, assessed in the present study, were richer in SFA and less abundant in PUFA and MUFA, is not in accordance with the previously reported outcomes in the apple ciders produced from fresh apple juices from the apple varieties Jonagold, Dabinett, and Aston Bitter, which were richer in PUFA and less abundant in SFA and MUFA [1]. However, as previously described, the mediums used for these apple cider fermentations were fresh apple juices (from the apple varieties Jonagold, Dabinett, and Aston Bitter, respectively), which were richer in PUFA and less abundant in MUFA [1].

On the other hand, the results obtained in the present study were comparable to those observed in PL from pasteurized apple juice [1], indicating that the increase of temperature during processing may be a reason for the reduction of PUFA and the increase of SFA. In addition, these outcomes further indicate that the commercially purchased apple juice that was already pasteurized and for ensuring that none contamination could occur it was further processed (autoclaved for at 121 °C for 15 min), which was used as the apple juice medium for the fermentations performed in the present study, could be the reason for observing less amounts of PUFA and more amounts of SFA in the PL bioactives from the apple ciders ACA, ACB, ACF, ACG, and ACI, assessed in the present study. This further suggest that if the apple juice, which is used as the medium for apple cider fermentations, was further been processed during concentration by higher temperatures before being used for any fermentation process, then such a preliminary concentrating process to the apple juice may have reduced the initial PUFA content and increase the SFA content of the PL bioactives in both the commercially purchased apple juice and the final apple cider fermented product.

Nevertheless, the presence of essential n-3 PUFA (mostly ALA, but also much lower but considerable amounts of EPA, DPA, and DHA) in the PL bioactives from all the novel functional apple ciders assessed in the present study, further support their anti-inflammatory and anti-platelet potency and provide a rationale for their strong anti-PAF and anti-ADP properties, which is also in accordance with previously observed relative outcomes in dietary PL of apple ciders [1] and from several other healthy beverages and food sources [3,4,18–23]. Such dietary PLs rich in n-3 PUFA have been found to inhibit platelet aggregation induced by the inflammatory and thrombotic mediators, PAF and thrombin, but also by classic well-established platelet agonists such as collagen and ADP [3,4,18–23], as was also observed in the present study for the rich in n-3 PUFA bioactive PL extracts of the apple ciders ACA, ACB, ACF, ACG, and ACI.

Interestingly, even though the PUFA content of all the apple ciders assessed in the present study were found to be lower from the relative PUFA levels previously observed in all the apple ciders produced from fresh apple juices of the apple varieties Jonagold, Dabinett, and Aston Bitter, yet the levels of the n-6/n-3 PUFA ratios observed in the PL bioactives of the apple ciders ACA, ACB, ACF, ACG, and ACI, were beneficially lower than the 3/1 value for this ratio, which is an index of the anti-inflammatory potency of dietary lipid bioactives [24]. It has been proposed that the lower the value for this ratio, the better the preventative anti-inflammatory benefits against several inflammation- and platelet aggregation-related chronic disorders, and vice versa [24]. Subsequently, the favorable low levels of the n-6/n-3 PUFA ratios observed in the PL bioactives of all the apple ciders assessed in the present study, which ranged approximately from 1.1 to 1.9 (Table 2), further support the potential anti-inflammatory and cardio-protective properties of the PL bioactives of the apple ciders ACA, ACB, ACF, ACG, and ACI, which were produced by fermenting apple juice incubated with the most bioactive and resilient/efficient for fermentations apple must yeast strains A, B, F, G, and I, respectively.

Moreover, the PL bioactives from both the apple ciders ACF and ACG showed statistically significant lowest values for their n-6/n-3 PUFA ratios and subsequently higher n-3 PUFA content, when compared to the PL from the other apple ciders. Taking also into account that the PL bioactives obtained from these two apple ciders showed the higher anti-inflammatory and anti-platelet potency against both the PAF and the ADP pathways, further suggest a structure activity relationship for these PL bioactives based on the concept tha the higher the n-3 PUFA content in PL bioactives (and thus the lower the relative n-6/n-3 PUFA ratio), the higher their anti-inflammatory and cardioprotective potency. In addition, these outcomes further support that the appropriate choosing of specific apple must yeast strains that are more bioactive and more resilient during apple cider fermentations, may increase the anti-platelet properties against PAF and ADP and subsequently to provide putative anti-inflammatory and cardioprotective functional properties of apple ciders. Nevertheless, more studies are needed in order to further support these promising outcomes.

4. Conclusions

Within the present study, specific bioassays based on a disease model of human platelet aggregation revealed that the PL, obtained from resilient and efficient for apple cider fermentation procedures apple must yeast strains, were more bioactive with strong anti-platelet properties. Moreover, by utilizing these most bioactive and resilient/efficient apple must yeast strains, several functional apple ciders were produced with PL bioactives that strongly inhibited platelet aggregation induced by PAF and ADP. The strong inhibition of the PL bioactives from these novel apple cider products against the inflammatory and thrombotic mediator PAF, in combination with their rich content in anti-inflammatory omega-3 PUFA like ALA and a subsequent favorable low omega-6/omega-3 PUFA ratio, further suggest putative anti-inflammatory and cardioprotective potency for these novel functional apple cider products.

Overall, these results further indicate that by choosing the more bioactive and resilient/efficient for fermentations yeast strains from apple must, then the anti-platelet functional properties of apple cider PL bioactives can be increased against thrombo-inflammatory mediators like PAF and ADP. Thus, these outcomes may facilitate the production of functional fermented products like apple cider, by valorizing the most optimum yeast strains, in terms of anti-platelet and anti-inflammatory potency and resilience/efficiency for fermentations. A wide range of applications for microbiological profiles of the fermentation industry through their yeast strains can thus improve the health characteristics and bioactive compound composition of alcoholic beverages like cider. As this is the first time that this has been researched a further in-depth study is required to accurately support the results and produce the parameters needed to efficiently characterize the apple must yeast strains (biochemical tests, sequencing, etc.) that can be utilized for increasing the bio-functional properties of their fermented cider products and their subsequent health benefits.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Faculty of Science & Engineering Research Ethics Committee (2017_10_10_S&E at 23 October 2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors. The data are not publicly available due to being assessed with specific softwares for aggregometry and GC-MS that are not available to anyone, while a licence is needed to use them.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ACA, ACB, ACF, ACG and ACI	fermented novel apple cider (AC) products derived from fermentations of apple juice by utilizing the most bioactive and resilient/efficient for fermentations apple must yeast strains A, B, F, G, and I, respectively.
PAF	platelet activating factor
CVD	cardiovascular diseases
ADP	adenosine 5′ diphosphate
BSA	bovine serum albumin
hPRP	human platelet-rich plasma
PL	polar lipids
TL	total lipids
NL	neutral lipids
GCMS	gas chromatography mass spectra
FAME	fatty acid methyl esters
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine

SFA	saturated fatty acids
MUFA	monounsaturated fatty acids
PUFA	polyunsaturated fatty acids
n-3	omega-3 PUFA
n-6	omega-6 PUFA
ALA	alpha linolenic acid
LA	linoleic acid
EPA	eicosapentaenoic acid
DPA	docosapentaenoic acid
DHA	docosahexaenoic acid
NA	nutrient agar
WLN	Wallerstein nutrient agar
MRS	De man, Rogosa, and Sharpe agar
WGA	wheat germ agglutinin
PBS	phosphate-buffered saline
PI	propidium iodide
ND	non-detectable

References

1. Tsoupras, A.; Moran, D.; Pleskach, H.; Durkin, M.; Traas, C.; Zabetakis, I. Beneficial Anti-Platelet and Anti-Inflammatory Properties of Irish Apple Juice and Cider Bioactives. *Foods* **2021**, *10*, 412. [CrossRef]
2. Tsoupras, A.; Moran, D.; Byrne, T.; Ryan, J.; Barrett, L.; Traas, C.; Zabetakis, I. Anti-Inflammatory and Anti-Platelet Properties of Lipid Bioactives from Apple Cider By-Products. *Molecules* **2021**, *26*, 2869. [CrossRef]
3. Fragopoulou, E.; Antonopoulou, S.; Tsoupras, A.; Tsantila, N.; Grypioti, A.; Gribilas, G.; Gritzapi, H.; Konsta, E.; Skandalou, E.; Papadopoulou, A.; et al. Antiatherogenic properties of red/white wine, musts, grape-skins, and yeast. *Chem. Phys. Lipids* **2004**, *130*, 66.
4. Tsoupras, A.; Lordan, R.; O'Keefe, E.; Shiels, K.; Saha, S.K.; Zabetakis, I. Structural Elucidation of Irish Ale Bioactive Polar Lipids with Antithrombotic Properties. *Biomolecules* **2020**, *10*, 1075. [CrossRef]
5. Tsoupras, A.B.; Demopoulos, C.A.; Pappas, K.M. Platelet-activating factor detection, metabolism and inhibitors in the ethanologenic bacterium Zymomonas mobilis. *Eur. J. Lipid Sci. Technol.* **2012**, *114*, 123–133. [CrossRef]
6. Le Quere, J.-M.; Husson, F.; Renard, C.M.G.C.; Primault, J. French cider charakterization by sensory, technological and chemical evaluations. *LWT Food Sci. Technol.* **2006**, *9*, 1033–1044. [CrossRef]
7. Al Daccache, M.; Koubaa, M.; Maroun, R.G.; Salameh, D.; Louka, N.; Vorobiev, E. Impact of the Physicochemical Composition and Microbial Diversity in Apple Juice Fermentation Process: A Review. *Molecules* **2020**, *25*, 3698. [CrossRef]
8. Ye, M.; Yue, T.; Yuan, Y. Effects of sequential mixed cultures of Wickerhamomyces anomalus and Saccharomyces cerevisiae on apple cider fermentation. *FEMS Yeast Res.* **2014**, *14*, 873–882. [CrossRef]
9. Morrissey, W.F.; Davenport, B.; Querol, A.; Dobson, A.D. The role of indigenous yeasts in traditional Irish cider fermentations. *J. Appl. Microbiol.* **2004**, *97*, 647–655. [CrossRef] [PubMed]
10. Lorenzini, M.; Simonato, B.; Slaghenaufi, D.; Ugliano, M.; Zapparoli, G. Assessment of yeasts for apple juice fermentation and production of cider volatile compounds. *LWT* **2019**, *99*, 224–230. [CrossRef]
11. Suarez Valles, B.; Bedrinana, R.P.; Tascon, N.F.; Simon, A.Q.; Madrera, R.R. Yeast species associated with the spontaneous fermentation of cider. *Food Microbiol.* **2007**, *24*, 25–31. [CrossRef] [PubMed]
12. Kennedy, D.; Cronin, U.P.; Wilkinson, M.G. Responses of Escherichia coli, Listeria monocytogenes, and Staphylococcus aureus to simulated food processing treatments, determined using fluorescence-activated cell sorting and plate counting. *Appl. Environ. Microbiol.* **2011**, *77*, 4657–4668. [CrossRef] [PubMed]
13. Herrero, M.; Quiros, C.; Garcia, L.A.; Diaz, M. Use of flow cytometry to follow the physiological states of microorganisms in cider fermentation processes. *Appl. Environ. Microbiol.* **2006**, *72*, 6725–6733. [CrossRef] [PubMed]
14. Ambriz-Aviña, V.; Contreras-Garduño, J.A.; Pedraza-Reyes, M. Applications of flow cytometry to characterize bacterial physiological responses. *Biomed. Res. Int.* **2014**, *2014*, 461941. [CrossRef]
15. Chaudhari, R.D.; Stenson, J.D.; Overton, T.W.; Thomas, C.R. Effect of bud scars on the mechanical properties of Saccharomyces cerevisiae cell walls. *Chem. Eng. Sci.* **2012**, *84*, 188–196. [CrossRef]
16. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917. [CrossRef]
17. Galanos, D.S.; Kapoulas, V.M. Isolation of polar lipids from triglyceride mixtures. *J. Lipid Res.* **1962**, *3*, 134–136. [CrossRef]
18. Tsoupras, A.; Lordan, R.; Demuru, M.; Shiels, K.; Saha, S.K.; Nasopoulou, C.; Zabetakis, I. Structural elucidation of irish organic farmed salmon (Salmo salar) polar lipids with antithrombotic activities. *Mar. Drugs* **2018**, *16*, 176. [CrossRef]
19. Tsoupras, A.; O'Keeffe, E.; Lordan, R.; Redfern, S.; Zabetakis, I. Bioprospecting for Antithrombotic Polar Lipids from Salmon, Herring, and Boarfish By-Products. *Foods* **2019**, *8*, 416. [CrossRef] [PubMed]

20. Tsoupras, A.; Zabetakis, I.; Lordan, R. Platelet aggregometry assay for evaluating the effects of platelet agonists and antiplatelet compounds on platelet function in vitro. *MethodsX* **2019**, *6*, 63–70. [CrossRef]
21. Tsoupras, A.; Lordan, R.; Harrington, J.; Pienaar, R.; Devaney, K.; Heaney, S.; Koidis, A.; Zabetakis, I. The Effects of Oxidation on the Antithrombotic Properties of Tea Lipids Against PAF, Thrombin, Collagen, and ADP. *Foods* **2020**, *9*, 385. [CrossRef] [PubMed]
22. Koukouraki, P.; Tsoupras, A.; Sotiroudis, G.; Demopoulos, C.A.; Sotiroudis, T.G. Antithrombotic properties of Spirulina extracts against platelet-activating factor and thrombin. *Food Biosci.* **2020**, *37*, 100686. [CrossRef]
23. Tsoupras, A.; Lordan, R.; Zabetakis, I. Inflammation, not Cholesterol, Is a Cause of Chronic Disease. *Nutrients* **2018**, *10*, 604. [CrossRef] [PubMed]
24. Simopoulos, A.P. The Importance of the Omega-6/Omega-3 Fatty Acid Ratio in Cardiovascular Disease and Other Chronic Diseases. *Exp. Biol. Med.* **2008**, *233*, 674–688. [CrossRef]