

## Article

# Chemical Characterization and Evaluation of Antioxidant, Anti-PAF and Anti-ADP Activities of Amphiphilic and Lipophilic Extracts from Caprine Milk and Commercial Kefir-Based Yogurt-Type Fermented Product

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## Abstract

Commercially available caprine dairy matrices are rich sources of lipid-associated bioactive constituents; however, comparative evaluations of their polarity-separated fractions remain limited. This study aimed to characterize and compare the chemical composition, antioxidant capacity, and inhibitory effects on thrombo-inflammatory agonist-induced platelet aggregation of amphiphilic (total amphiphilic content, TAC) and lipophilic (total lipophilic content, TLC) fractions isolated from three independently produced commercial goat milk and three independently produced commercial kefir-based goat yogurt-type fermented products, with different LOT numbers. Total lipid (lipophilic and amphiphilic) extracts were obtained by Bligh and Dyer extraction and then fractionated to TAC and TLC by counter-current separation using a green hexane/ethanol–water solvent system. Analytical determinations included spectrophotometric quantification of total carotenoid and phenolic contents, attenuated total reflectance Fourier-transform infrared (ATR–FTIR) structural profiling, and liquid chromatography–mass spectrometry (LC–MS) relative fatty-acid profiling. Antioxidant capacity was assessed using DPPH and ABTS radical-scavenging assays, while ex vivo light-transmission aggregometry in human platelet-rich plasma, obtained from six independent healthy donors ( $n = 6$ ) for each fraction, was employed to evaluate potential inhibitory effects against platelet-activating factor (PAF) and adenosine diphosphate (ADP). Across both matrices, TAC fractions exhibited markedly higher radical-scavenging responses than the corresponding TLC fractions, particularly in the ABTS assay. In platelet aggregometry, TAC fractions showed significantly stronger inhibition of both PAF- and ADP-induced aggregation than their corresponding TLC fractions. Specifically, the TAC fraction from the kefir-based yogurt product exhibited the lowest  $IC_{50}$  value against PAF-induced aggregation, whereas the two TAC fractions showed comparable inhibitory potency against ADP-induced aggregation. ATR–FTIR spectroscopy revealed spectral features consistent with lipid-associated, hydroxyl-containing, and phosphate-containing amphiphilic structures, while LC–MS profiling identified palmitic, stearic, oleic, and  $\alpha$ -linolenic acids among the predominant fatty acids in the free and esterified fatty-acid pools. Overall, the TAC fractions exhibited substantial in vitro radical-scavenging activity and ex vivo inhibition of agonist-induced platelet aggregation, suggesting potential anti-



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inflammatory and antithrombotic relevance for these activities. Further in vivo studies are required to determine their physiological relevance.

**Keywords:** goat milk; commercial kefir yogurt; amphiphilic bioactives; polar lipids; antioxidant activity; platelet aggregation; PAF; ADP

## 1. Introduction

Fermented dairy products represent complex matrices containing bioactive compounds, some of which are generated or modified through microbial metabolism, proteolysis, and lipolysis [1]. Among these, kefir—a product of symbiotic fermentation by lactic acid bacteria, acetic acid bacteria, and yeasts—has attracted significant research interest because of its potential health-promoting properties [2,3]. Small-ruminant milks, particularly caprine milk, exhibit distinct nutritional and biochemical characteristics compared with bovine milk, including smaller fat globule sizes, enhanced digestibility, and a distinct lipid architecture [2–4]. When used as a substrate for fermented dairy products, goat milk undergoes compositional changes that may affect its lipid and amphiphilic constituents [4].

Dairy lipids comprise neutral lipids, predominantly triacylglycerols, and polar/amphiphilic lipids such as phospholipids and glycolipids, together with associated bioactive constituents [5]. Polar and amphiphilic lipids are integral components of biological membranes and the milk fat globule membrane (MFGM) [6]. These fractions have attracted increasing interest because of their reported radical-scavenging activity and potential involvement in receptor-mediated signaling pathways [7–10]. Platelet activation plays an important role in thrombo-inflammatory processes. Platelet-activating factor (PAF) is a potent phospholipid mediator involved in inflammatory signaling and platelet activation, whereas adenosine diphosphate (ADP) is a key agonist of platelet aggregation.

Although unfractionated dairy extracts have been evaluated for general biological properties, the specific contributions of polarity-separated amphiphilic (total amphiphilic content, TAC) and lipophilic (total lipophilic content, TLC) fractions remain insufficiently characterized. Furthermore, the available literature rarely combines functional radical-scavenging and agonist-specific platelet-aggregation assays with structural characterization by attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) and relative fatty-acid profiling by liquid chromatography–mass spectrometry (LC-MS). Importantly, comparisons involving commercial dairy matrices—such as commercial liquid goat milk and a commercial kefir-based yogurt-type fermented product—require careful interpretation. Because commercial products originate from distinct processing streams, starter cultures, and batch formulations, direct comparisons between them cannot be interpreted as a controlled assessment of fermentation-induced changes in a single starting milk. Instead, such evaluations provide an exploratory comparison of the bioactive profiles of distinct commercial caprine dairy matrices.

To address these knowledge gaps, the primary objective of this study was to systematically isolate, characterize, and compare the amphiphilic (TAC) and lipophilic (TLC) fractions obtained from two commercial caprine dairy matrices: commercial goat milk and a commercial kefir-based yogurt-type fermented product. Specifically, this study aimed to:

- i. Evaluate and compare the in vitro antioxidant capacity, using DPPH and ABTS radical-scavenging assays, and the ex vivo inhibition of PAF- and ADP-induced platelet aggregation by TAC and TLC fractions in human platelet-rich plasma (hPRP;  $n = 6$  independent donors).

- ii. Perform a secondary, exploratory comparison of these bioactive profiles between the two commercial caprine dairy matrices.
- iii. Profile total carotenoid and phenolic contents, characterize major functional groups using ATR–FTIR spectroscopy, and determine the relative abundances of free and esterified fatty acids using LC–MS.

## 2. Materials and Methods

### 2.1. Materials

Three independently produced commercial goat milk and three independently produced commercial kefir-based goat yogurt-type fermented products with different LOT numbers were purchased from a local market in Greece. High-performance liquid chromatography (HPLC)-grade solvents (n-hexane, ethanol, methanol, chloroform, and water) were obtained from Merck (Darmstadt, Germany). Synthetic platelet-activating factor (PAF; 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine), adenosine 5'-diphosphate (ADP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), gallic acid, and  $\beta$ -carotene standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

Solvent removal was performed using a Büchi rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). Spectrophotometric analyses were conducted with a uniSPEC 2 UV–Vis spectrophotometer (LLG Labware, Meckenheim, Germany). Structural characterization of selected extracts was performed using attenuated total reflectance Fourier-transform infrared spectroscopy (ATR–FTIR) with a Frontier FT-IR spectrometer (PerkinElmer, Waltham, MA, USA).

### 2.2. Extraction and Separation of Amphiphilic and Lipophilic Fractions from Goat Milk and the Kefir-Based Yogurt-Type Product

Total lipid extracts containing both amphiphilic and lipophilic compounds were isolated from both commercial dairy matrices using a modified Bligh and Dyer procedure. Briefly, homogeneous samples of goat milk and kefir-based yogurt were homogenized with chloroform/methanol/water (1:2:0.8, *v/v/v*). Phases were separated by adjusting the final solvent ratio to 2:2:1.8 (*v/v/v*) via the addition of chloroform and water. The lower organic phase containing total lipids was collected and evaporated to dryness using a rotary evaporator at 37 °C.

To separate the total extract into total amphiphilic content (TAC) and total lipophilic content (TLC) fractions, a counter-current liquid–liquid extraction was performed using a green n-hexane/ethanol–water (10:9:1, *v/v/v*) biphasic solvent system [10], according to Directive 2009/32/EC. The upper hexane-rich phase yielded the TLC fraction (predominantly neutral lipids and non-polar constituents), while the lower aqueous-ethanolic phase yielded the TAC fraction (enriched in polar lipids and amphiphilic constituents, including phenolics and several carotenoids). Both fractions were evaporated to dryness under a stream of nitrogen, weighed gravimetrically to establish yield, and stored at –20 °C under argon prior to analysis.

### 2.3. Yield of Extraction

The extraction yields of the total extract and the corresponding TAC and TLC fractions were determined gravimetrically from the dry mass obtained after solvent removal.

For goat milk, yields were expressed as grams of dry extract per 100 g of milk, whereas for the kefir-based yogurt-type fermented product, yields were expressed as grams of dry extract per 100 g of product. The yield was calculated using the mass of the dried extract relative to the initial sample mass.

All yield measurements were performed using the three independent extraction replicates per commercial matrix, according to the following equations:

$$\text{Product extraction yield (g/100 g)} = [\text{mass of dry extract (g)} / \text{initial sample mass (g)}] \times 100$$

The yield for TL was calculated separately for each independent commercial lot from its paired TAC and TLC values before any statistical summarization was performed.

#### 2.4. Quantification of Total Phenolic Content

Total phenolic content (TPC) was determined using the Folin–Ciocalteu assay according to previously reported methodology [4,10].

Briefly, an aliquot of each extract was mixed with 1 mL deionized water and 1 mL Folin–Ciocalteu reagent. After 7 min, 3 mL of sodium carbonate solution was added. The reaction mixture was incubated in the dark at room temperature for 2 h, with intermittent vortex mixing. Absorbance was measured at 765 nm.

TPC was calculated using a gallic acid calibration curve and expressed as mg gallic acid equivalents (GAE) per g dry weight of extract/fraction. Analytical measurements were performed in triplicate. The TPC for TL was calculated separately for each independent commercial lot from its paired TAC and TLC values before any statistical summarization was performed.

#### 2.5. Quantification of Total Carotenoid Content

Total carotenoid content (TCC) was determined spectrophotometrically using  $\beta$ -carotene as the reference standard, following previously described methodology [10].

Each sample was dissolved in 2 mL hexane and absorbance was measured at 450 nm. TCC was calculated using a  $\beta$ -carotene calibration curve and expressed as mg  $\beta$ -carotene equivalents (CE) per g dry weight of extract. Analytical determinations were performed in triplicate. The TCC for TL was calculated separately for each independent commercial lot from its paired TAC and TLC values before any statistical summarization was performed.

#### 2.6. Total Antioxidant Activity (TAA)

Antioxidant activity was assessed using complementary DPPH and ABTS radical-scavenging assays [4,10].

##### 2.6.1. DPPH Radical-Scavenging Assay

An aliquot of each extract was mixed with 0.2 mL ethanol, 0.8 mL Tris–HCl buffer (pH 7.4), and 1 mL DPPH solution. The mixtures were vortexed following the addition of each reagent and incubated at room temperature for 30 min. Absorbance was measured at 517 nm using the following equation:

$$\text{Inhibition (\%)} = (A1 - A2) / A1 \times 100$$

Radical-scavenging activity was calculated relative to the control. The  $IC_{50}$  was defined as the extract/fraction concentration required to inhibit 50% of the initial DPPH radical response. Trolox was used as the reference antioxidant, and the corresponding Trolox equivalent antioxidant capacity (TEAC) was calculated from the ratio of the Trolox  $IC_{50}$  to the sample  $IC_{50}$ , as follows:

$$TEAC = IC_{50} \text{ of Trolox } (\mu\text{g/L}) / IC_{50} \text{ of the sample } (\mu\text{g/L})$$

The TEAC values for TL was calculated separately for each independent commercial lot from its paired TAC and TLC values before any statistical summarization was performed.

### 2.6.2. ABTS Radical-Scavenging Assay

For the ABTS assay, an aliquot of each extract/fraction was mixed with 2 mL ABTS radical solution, vortexed, and incubated in the dark for 7 min at room temperature. Absorbance was measured at 734 nm.

Trolox was used as the reference standard. Antioxidant capacity was expressed as  $\mu\text{mol}$  Trolox equivalents (TE) per g dry weight of extract/fraction, according to the following equation:

$$ABTS (\mu\text{molTE/gDW}) = (c \times V \times t)/m$$

where  $c$  is the Trolox-equivalent concentration obtained from the calibration curve,  $V$  is the sample volume (mL),  $t$  is the dilution factor, and  $m$  is the dry mass of the sample (g). The ABTS values for TL was calculated separately for each independent commercial lot from its paired TAC and TLC values before any statistical summarization was performed.

### 2.7. Quantification of the Anti-Inflammatory and Antithrombotic Efficacy by the Platelet Aggregometry Assay

Platelet-aggregation experiments were performed to evaluate the capacity of the goat milk- and kefir-based yogurt-type product fractions to inhibit platelet aggregation induced by platelet-activating factor (PAF) and adenosine diphosphate (ADP). The assay was performed using human platelet-rich plasma (hPRP) from blood samples in citrate, obtained from six independent healthy adult donors per fraction, in a Chrono-log 490 four-channel strobilometric platelet aggregometer (Havertown, PA, USA), connected to the accompanying AGGRO/LINK<sup>®</sup>8 (Version 8.1.2.2) program software package, as previously described by Kosidou et al. [11].

Eligible participants were healthy adults without a history of cardiovascular disease and without medication known to affect platelet function. All participants provided written informed consent before blood collection. The study protocol was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Democritus University of Thrace (protocol codes  $\Delta\Pi\Theta/\text{EH}\Delta\text{E}/7690/70$ ; approval date: 27 September 2024 and  $\Delta\Pi\Theta/\text{EH}\Delta\text{E}/73216/640$ ; approval date: 24th June 2026). The experimental study extended across the periods covered by these successive approvals, and all blood collection and platelet experiments were performed under a valid ethical approval in force at the time of experimentation.

Each extract was tested at multiple concentrations to generate concentration–response curves. hPRP was pre-incubated with the respective extract/fraction before addition of PAF or ADP. In the absence of extract, the selected agonist concentration produced maximal reversible platelet aggregation and was defined as the positive control corresponding to 100% aggregation. Unstimulated hPRP served as the negative control.

Platelet aggregation was monitored by changes in light transmission using a platelet aggregometer. Extract concentrations were selected within the concentration range corresponding to 20–80% of maximal reversible aggregation. The  $\text{IC}_{50}$  was calculated from the concentration–response relationship and expressed as the mass of dry extract ( $\mu\text{g}$ ) required to inhibit 50% of agonist-induced platelet aggregation. Lower  $\text{IC}_{50}$  values therefore indicate greater inhibitory potency under the experimental conditions employed.

Platelet responses were determined separately for each of the three independently prepared extracts per experimental group, originating from three different commercial lots of each dairy matrix. Each extract was evaluated in hPRP from six different healthy donors, generating six donor-level  $\text{IC}_{50}$  values per extract and 18 individual  $\text{IC}_{50}$  determinations per experimental group. Each  $\text{IC}_{50}$  therefore represents the response of one donor's hPRP to one specific extract preparation; extracts were neither pooled nor averaged prior to platelet testing, and different donor sets were used for the three extract preparations. Thus,

the biological experimental unit was the individual blood donor, whereas extract preparations and analytical replicate measurements were not treated as additional independent biological observations.

## 2.8. ATR–FTIR-Based Structural Analysis of TAC Extracts from Goat Milk and the Kefir-Based Yogurt-Type Product

The functional groups present in the TAC fractions were characterized using ATR–FTIR spectroscopy, following previously reported procedures [4,10].

Spectra were acquired using a PerkinElmer Frontier ATR/FT-NIR/MIR spectrophotometer (PerkinElmer, Shelton, CT, USA) over the spectral range of 600–4000  $\text{cm}^{-1}$ , at a resolution of 1  $\text{cm}^{-1}$ , with 3 scans accumulated per spectrum. A sufficient quantity of each dried TAC fraction was placed directly onto the ATR crystal to ensure complete contact, and spectra were recorded after stabilization of the instrumental signal under identical acquisition conditions for all samples. Three independently prepared TAC samples/three replicate measurements were analyzed for each dairy matrix. No additional spectral preprocessing was applied beyond the actual procedure.

Reference spectra of quercetin, catechin, gallic acid,  $\beta$ -carotene, and soybean-derived polar lipids were acquired under the same instrumental conditions to support comparative band assignment. The ATR crystal was cleaned with isopropanol between measurements, and isopropanol was additionally analyzed under the same conditions to identify potential solvent-related spectral contributions.

## 2.9. LC–MS Analysis of Fatty-Acid Profiles

The fatty-acid profiles of the TAC fractions were investigated before and after saponification using LC–MS according to previously described procedures [4,10].

Dried TAC samples were reconstituted in 500  $\mu\text{L}$  dichloromethane/methanol (1:2,  $v/v$ ) and centrifuged at 13,000 rpm for 6 min. The supernatants were passed through 3 kDa ultrafiltration membranes (Amicon Ultra 3K, Merck Millipore, Darmstadt, Germany). A 10  $\mu\text{L}$  aliquot of each filtrate was injected into an Agilent 1260 HPLC system (Agilent, Santa Clara, CA, USA) coupled to an Agilent 6520 Q-TOF mass spectrometer operating in negative ESI mode.

Chromatographic separation was performed using an Agilent Poroshell 120 EC-C18 column (2.7  $\mu\text{m}$ , 3.0  $\times$  150 mm). The mobile phases consisted of 2 mM ammonium acetate in water (Phase A) and 2 mM ammonium acetate in 95% acetonitrile (Phase B). The chromatographic elution program was as follows: [INSERT ACTUAL %A/%B versus time PROGRAM]. The flow rate was 0.3 mL/min during the first 5 min and was subsequently increased to 0.6 mL/min [add total run time, if available]. Mass spectra were acquired over an  $m/z$  range of 50–1100.

The instrumental parameters were as follows: capillary voltage, 3500 V; skimmer voltage, 65 V; fragmentor voltage, 175 V; drying gas flow, 5 L/min; nebulizer pressure, 30 psi; and drying gas temperature, 325  $^{\circ}\text{C}$ . Fatty-acid identities were evaluated by comparison with external standards, including C12:0, C14:0, C16:0, C18:0, C18:1n-9, C18:2n-6, and C18:3n-3.

Fatty-acid profiles were evaluated semiquantitatively based on normalized LC–MS peak areas obtained from triplicate analyses. The relative abundance of each detected fatty acid was expressed as its normalized peak-area contribution to the total detected fatty-acid peak area. As compound-specific internal standards and response-factor corrections were not employed, these values represent semiquantitative relative abundances and not absolute fatty-acid concentrations. Triplicate measurements were used to assess the consistency of the analytical determinations; no separate formal analytical-method validation or dedicated repeatability study was performed as part of the present investigation.

### 2.10. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 29.0 (IBM Corp., Armonk, NY, USA). Analytical determinations were performed on three independently produced commercial lots per dairy matrix ( $n = 3$ ), with analytical replicates performed as specified for the respective assays.

For the platelet-aggregation experiments, each of the three independently prepared extracts per experimental group was evaluated using hPRP obtained from six different independent healthy donors. Different donor sets were used for each extract preparation; thus, the same donors were not repeatedly tested across the different extract preparations. Accordingly, 18 individual donor-level IC<sub>50</sub> values were obtained per experimental group (3 independently prepared extracts  $\times$  6 different donors per extract).

Data distribution was assessed using the Shapiro–Wilk/Kolmogorov–Smirnov tests. Parametric data, including the individual IC<sub>50</sub> values from the platelet-aggregation experiments, were analyzed using one-way ANOVA followed by Tukey’s HSD post hoc test for multiple comparisons. Non-parametric data were analyzed using the Kruskal–Wallis test followed by appropriate pairwise comparisons. Statistical significance was defined as  $p < 0.05$ .

Parametric data are reported as mean  $\pm$  SD, whereas non-parametric data are reported as median with minimum and maximum values.

## 3. Results

### 3.1. Yield of Extraction

Extraction yields differed numerically among the fractions, although the overall difference was not statistically significant (one-way ANOVA,  $p = 0.137$ ). The mean yields were 6.29 g/100 g for the TAC fraction of the kefir-based goat yogurt-type fermented product (yTAC), 5.18 g/100 g for goat milk TAC (mTAC), 7.33 g/100 g for yogurt TLC (yTLC), and 9.98 g/100 g for milk TLC (mTLC). Within both dairy matrices, the TLC fractions showed numerically higher extraction yields than the corresponding TAC fractions, with mTLC exhibiting the highest mean yield. However, the Tukey post hoc test detected no significant pairwise differences (all  $p > 0.05$ ). The observed pattern is consistent with the general lipid composition of dairy products, in which neutral lipids constitute the predominant lipid pool, whereas amphiphilic fractions contain a smaller proportion of polar lipids [12], together with minor amphiphilic bioactives such as carotenoids and phenolic compounds [13,14]. Previous lipidomic comparisons have associated cow milk mainly with triglyceride-rich neutral lipid profiles, whereas mare’s milk has shown a greater contribution from polar and membrane-related lipids [15]. Goat milk follows a broadly similar compositional pattern to cow milk, with neutral lipids accounting for approximately 95–99% and polar lipids for approximately 1–5% of the total lipid content [12]. The slightly higher yTAC yield compared with mTAC may reflect matrix-dependent differences in the extractable amphiphilic pool following fermentation; nevertheless, this interpretation should be considered tentative because the observed difference was not statistically significant [16]. Importantly, extraction yield alone did not parallel biological activity. Despite their lower recovered mass, the TAC fractions exhibited stronger antioxidant activity than the corresponding TLC fractions, supporting previous observations that the biological performance of an extract depends on its composition rather than on extraction yield alone [17].

Nevertheless, it should also be stressed that the reported extraction yields primarily describe the recovery obtained from each commercial matrix under the applied extraction conditions and should not be interpreted as a dry-matter- or lipid-normalized comparison of extraction efficiency between goat milk and the kefir-based yogurt-type product.

### 3.2. Total Phenolic and Carotenoid Content of Kefir Milk and Yogurt

#### 3.2.1. Carotenoid Content

The total carotenoid content (TCC), expressed as mg  $\beta$ -carotene equivalents (CE) per g dry weight (DW) of extract, differed significantly among the four extract groups (Kruskal–Wallis:  $H = 8.744$ ,  $df = 3$ ,  $p = 0.033$ ; Table 1). At the descriptive level, the goat milk amphiphilic fraction (mTAC) showed the highest median TCC (3.137 mg CE/g DW), with values ranging from 1.887 to 7.060 mg CE/g DW, indicating a relatively wide within-group range. The yogurt amphiphilic fraction (yTAC) had a median of 0.873 mg CE/g DW and a range of 0.545–2.214 mg CE/g DW. A similar median was observed for the yogurt lipophilic fraction (yTLC; 0.846 mg CE/g DW; range: 0.628–1.075 mg CE/g DW), whereas the milk lipophilic fraction (mTLC) showed the lowest median value (0.153 mg CE/g DW; range: 0.088–0.202 mg CE/g DW). Thus, mTAC presented the highest median TCC, yTAC and yTLC displayed comparable median values, and mTLC showed the lowest TCC. The possible contribution of fermentation-related changes to this pattern is considered in the Discussion [13].

**Table 1.** Total carotenoid content of TAC, TLC, and TL extracts from goat milk and a kefir-based goat yogurt-type fermented product. Results are expressed as mg  $\beta$ -carotene equivalents (CE) per g dry weight (DW) of extract (analyses were performed in extracts from three independently produced commercial lots for each dairy matrix).

| Milk       |            |           | Yogurt     |            |           |
|------------|------------|-----------|------------|------------|-----------|
| TCC of TAC | TCC of TLC | TCC of TL | TCC of TAC | TCC of TLC | TCC of TL |
| Median     | Median     | Median    | Median     | Median     | Median    |
| 3.14       | 0.15       | 3.29      | 0.87       | 0.84       | 1.71      |
| Max        | Max        | Max       | Max        | Max        | Max       |
| 7.06       | 0.20       | 7.26      | 2.21       | 1.07       | 3.28      |
| Min        | Min        | Min       | Min        | Min        | Min       |
| 1.88       | 0.08       | 1.96      | 0.55       | 0.63       | 1.18      |

TCC = total carotenoid content, TAC = total amphiphilic compounds, TLC = total lipophilic compounds, TL = total lipids, CE =  $\beta$ -carotene equivalents, DW = dry weight.

#### 3.2.2. Phenolic Content

Under the applied assay conditions, total phenolic content (TPC) was not detected in the unsaponified amphiphilic fractions (mTAC and yTAC). In contrast, measurable TPC values were obtained for the lipophilic fractions (mTLC and yTLC), ranging from 0.06 to 0.48 mg gallic acid equivalents (GAE)/g dry weight (DW). The overall comparison among groups was borderline according to the Kruskal–Wallis test ( $p = 0.050$ ), with yTLC showing a higher median TPC than mTLC. The median, maximum, and minimum values for the amphiphilic and lipophilic fractions are presented in Table 2. These findings show that Folin–Ciocalteu-reactive compounds were measurable only in the lipophilic fractions under the conditions used; their potential chemical origin and relationship with fermentation are examined in the Discussion [18].

**Table 2.** Total phenolic content (TPC) of TAC, TLC, and TL extracts from goat milk and a kefir-based goat yogurt-type fermented product. Results are expressed as mg gallic acid equivalents (GAE) per g dry weight (DW) of extract (analyses were performed on extracts from three independently produced commercial lots for each dairy matrix).

| Milk       |            |           | Yogurt     |            |           |
|------------|------------|-----------|------------|------------|-----------|
| TPC of TAC | TPC of TLC | TPC of TL | TPC of TAC | TPC of TLC | TPC of TL |
| Median     | Median     | Median    | Median     | Median     | Median    |
| ND         | 0.17       | 0.17      | ND         | 0.37       | 0.37      |
| Max        | Max        | Max       | Max        | Max        | Max       |
| ND         | 0.20       | 0.20      | ND         | 0.48       | 0.48      |
| Min        | Min        | Min       | Min        | Min        | Min       |
| ND         | 0.10       | 0.10      | ND         | 0.27       | 0.27      |

TAC = total amphiphilic compounds, TLC = total lipophilic compounds, TL = total lipids, DW = dry weight, TPC = total phenolic content; GAE = gallic acid equivalents, ND = not detected under the applied assay conditions.

### 3.3. Antioxidant Activities

The antioxidant potential of TAC, TLC, and TL extracts from goat milk and from a kefir-based goat yogurt-type fermented product was assessed using ABTS and DPPH assays. Results were expressed as  $\mu\text{mol}$  Trolox equivalents (TE)/g dry weight (DW) for the ABTS assay and as Trolox equivalent antioxidant capacity (TEAC) values for the DPPH assay. Depending on data distribution, group comparisons were performed using either one-way ANOVA or the Kruskal–Wallis test, followed by the appropriate post hoc analysis.

#### 3.3.1. ABTS Radical Scavenging Activity

The ABTS<sup>+</sup> scavenging results differed significantly among the four extract groups (Kruskal–Wallis:  $H = 8.436$ ,  $df = 3$ ,  $p = 0.038$ ). As shown in Table 3, the TAC fractions displayed higher ABTS values than the corresponding TLC fractions. Milk TAC (mTAC) showed a range of 0.413–3.736  $\mu\text{mol}$  TE/g DW and a median of 1.943  $\mu\text{mol}$  TE/g DW, reflecting a relatively wide within-group range. Yogurt TAC (yTAC) displayed a narrower range of 0.562–2.191  $\mu\text{mol}$  TE/g DW, with a median of 1.746  $\mu\text{mol}$  TE/g DW. No statistically significant difference was detected between mTAC and yTAC ( $p = 0.910$ ). The TLC fractions showed lower ABTS values. Yogurt TLC (yTLC) ranged from 0.068 to 0.147  $\mu\text{mol}$  TE/g DW, with a median of 0.127  $\mu\text{mol}$  TE/g DW, whereas milk TLC (mTLC) ranged from 0.089 to 0.099  $\mu\text{mol}$  TE/g DW, with a median of 0.089  $\mu\text{mol}$  TE/g DW. The higher ABTS activity observed in the TAC fractions is considered further in the Discussion [19].

**Table 3.** Antioxidant capacity of TAC, TLC, and TL extracts from goat milk and a kefir-based goat yogurt-type fermented product, as determined by the ABTS assay. Results are expressed as  $\mu\text{mol}$  Trolox equivalents (TE)/g dry weight (DW) (analyses were performed on extracts from three independently produced commercial lots for each dairy matrix).

| ABTS Values |        |      |      |
|-------------|--------|------|------|
| Milk        | Median | Max  | Min  |
| TAC         | 1.94   | 3.74 | 0.41 |
| TLC         | 0.09   | 0.10 | 0.09 |
| TL          | 2.03   | 3.84 | 0.50 |
| Yogurt      | Median | Max  | Min  |
| TAC         | 1.74   | 2.19 | 0.56 |
| TLC         | 0.13   | 0.15 | 0.07 |
| TL          | 1.87   | 2.34 | 0.63 |

TAC = total amphiphilic compounds, TLC = total lipophilic compounds, TL = total lipids, DW = dry weight.

### 3.3.2. DPPH Radical Scavenging Activity

The antioxidant activity of the extracts was evaluated using the DPPH radical scavenging assay. TEAC values were calculated as the ratio of the IC<sub>50</sub> of Trolox (µg/mL) to the IC<sub>50</sub> of each sample (µg/mL) for mTAC, yTAC, yTLC, and mTLC. As presented in Table 4, the TAC fractions showed higher TEAC values than the corresponding TLC fractions. The mTAC fraction had a median TEAC value of 0.62, with values ranging from 0.26 to 0.90. The yTAC fraction showed a median of 0.39 and a wider range of 0.22–5.34. In contrast, yTLC showed a median TEAC value of 0.02, with a range of 0.017–0.024, while mTLC also had a median of 0.02, with values ranging from 0.014 to 0.016. Post hoc pairwise comparisons yielded unadjusted *p*-values of 0.013 for yTLC versus yTAC and 0.009 for mTLC versus mTAC. The remaining comparisons were not statistically significant, with adjusted *p*-values greater than 0.3. Because the significance of pairwise comparisons depends on the correction applied for multiple testing, these results should be interpreted accordingly. The higher TEAC values observed for the TAC fractions are discussed in relation to previous findings [20].

**Table 4.** Antioxidant capacity of TAC, TLC, and TL extracts from goat milk and a kefir-based goat yogurt-type fermented product, as determined by the DPPH assay. Results are expressed as Trolox equivalent antioxidant capacity (TEAC) values (analyses were performed on extracts from three independently produced commercial lots for each dairy matrix).

| TEAC Values |        |      |      |
|-------------|--------|------|------|
| Milk        | Median | Max  | Min  |
| TAC         | 0.62   | 0.90 | 0.26 |
| TLC         | 0.02   | 0.02 | 0.01 |
| TL          | 0.63   | 0.92 | 0.27 |
| Yogurt      | Median | Max  | Min  |
| TAC         | 0.39   | 5.34 | 0.22 |
| TLC         | 0.02   | 0.02 | 0.02 |
| TL          | 0.42   | 5.36 | 0.23 |

TAC = total amphiphilic compounds, TLC = total lipophilic compounds, TL = total lipids.

### 3.4. FT-IR Spectrum Analysis (TAC Goat Milk and Yogurt Extracts)

ATR-FTIR analysis was performed on six amphiphilic (TAC) extracts, comprising three replicate extracts from goat milk and three from the kefir-based goat yogurt-type fermented product. The spectra showed broadly comparable profiles, with only minor differences in band intensity between the two sample types. In all spectra, a broad band was observed at 3200–3600 cm<sup>−1</sup> and assigned to O–H stretching vibrations. Because this region may arise from several hydroxyl-containing compounds and hydrogen-bonded species, it was considered indicative of hydroxyl-bearing constituents rather than a compound-specific marker. Bands in the 3000–2850 cm<sup>−1</sup> region were assigned to asymmetric and symmetric stretching vibrations of –CH<sub>2</sub> and –CH<sub>3</sub> groups, consistent with aliphatic chains. Carbonyl absorptions within 1750–1700 cm<sup>−1</sup> were associated with ester and carboxylic acid groups. Together, these bands support the occurrence of lipid-derived aliphatic and carbonyl functionalities in the TAC extracts but do not, by themselves, permit identification of individual lipid classes.

Absorptions within 1610–1500 cm<sup>−1</sup> were attributed to overlapping aromatic C=C and protein/peptide-related vibrations, while bands at 1470–1390 cm<sup>−1</sup> were consistent with CH<sub>2</sub>/CH<sub>3</sub> deformation and carboxylate-related modes. The 1260–1220 cm<sup>−1</sup> region was assigned to phosphate-related P=O and P–O–C vibrations, and the 1200–1000 cm<sup>−1</sup> region contained C–O–C, C–O, C–O–P, and glycosidic vibrations. These features are compatible

with the presence of phospholipid-, glycolipid-, carbohydrate-, and other amphiphilic structures. The fingerprint region ( $1500\text{--}600\text{ cm}^{-1}$ ) contained several overlapping bands and therefore was interpreted cautiously. The yogurt-derived TAC spectra showed slightly greater band intensities in parts of the O–H and C–O regions than the milk-derived TAC spectra; however, no quantitative conclusion regarding compound abundance was drawn from these intensity differences. Overall, the spectra were consistent with a chemically heterogeneous amphiphilic matrix containing lipid-related, hydroxyl-bearing, carbohydrate-related, and protein/peptide-associated functional groups [18,21]. The principal bands and their tentative assignments are summarized in Table 5.

**Table 5.** Characteristic peaks of TAC fractions from goat milk and kefir yoghurt-type samples in ATR-FTIR and their chemical interpretation in relation to phenolic compounds, carotenoids and protein/peptide structures (analyses were performed on extracts from three independently produced commercial lots for each dairy matrix).

| Region ( $\text{cm}^{-1}$ ) | Peaks | Characteristic Bonds                                 | Bioactive Components of Goat Milk                                        |
|-----------------------------|-------|------------------------------------------------------|--------------------------------------------------------------------------|
| 3600–3200                   | ~3350 | Stretching O-H                                       | Phenolic compounds, alcohols, carbohydrates, peptides, hydrophilic bonds |
| 3000–2850                   | ~     | Stretching C-H ( $-\text{CH}_2$ , $-\text{CH}_3$ )   | Alkyl chain, fatty acids, phospholipids, triglycerides                   |
| 1750–1730                   | ~     | C=O of ester groups                                  | Estered fatty acids                                                      |
| 1720–1700                   | ~     | C=O of carboxyl acids                                | Free fatty acids, phenolic acids                                         |
| 1680–1630                   | ~     | C=O, C=C of proteins                                 | Amide I band (e.g., carotenoids)                                         |
| 1560–1515                   | ~     | N-H bending, C-N stretching                          | Amide II band from milk peptides                                         |
| 1610–1500                   | ~     | Stretching C=C aromatic ring                         | Indicative of phenolic and flavonoid structures                          |
| 1470–1450                   | ~     | Bending $\text{CH}_2/\text{CH}_3$                    | Alkyl chains of fatty acids                                              |
| 1420–1390                   | ~     | Symmetrical stretching<br>COO-/bending $\text{CH}_2$ | Organic acids, fatty acids                                               |
| 1260–1220                   | ~     | P=O, P-O-C (phosphodiester)                          | Polar lipids, phospholipids                                              |
| 1200–1100                   | ~     | C-O-C, C-O bending                                   | Esters, alcohols, glycolipids, phospholipids                             |
| 1100–1000                   | ~     | C-O, C-O-P, glycosidic bonds                         | Unsaturated fatty acids                                                  |
| 970–600                     | ~     | C-H fingerprint region                               | Phenolics, lipids, proteins, carbohydrates                               |

### 3.5. Semiquantitative LC–MS Derived Fatty-Acid Profile of the TAC Fractions from Goat Milk and Goat Yogurt-Type Kefir Product

LC–MS analysis was used to characterize the fatty acid profiles of the saponified and non-saponified TAC fractions obtained from goat milk (mTAC) and from a kefir-based yogurt-type fermented product (yTAC). The results for the saponified fractions are presented first in Table 6, followed by the results for the non-saponified fractions in Table 7. In the saponified mTAC fraction, palmitic acid (C16:0) and stearic acid (C18:0) were the predominant fatty acids, accounting for  $33.73 \pm 0.25\%$  and  $51.06 \pm 0.20\%$ , respectively. Oleic acid (C18:1 n-9) was present at a substantially lower relative abundance ( $2.92 \pm 0.08\%$ ) (Table 6). A similar fatty-acid profile was observed for the yTAC fraction, with palmitic acid ( $33.78 \pm 0.07\%$ ), stearic acid ( $51.12 \pm 0.13\%$ ), and oleic acid ( $2.91 \pm 0.07\%$ ) being again the most abundant listed fatty acids. The remaining fatty acids were detected at lower levels or were not detected. The fatty acid category totals reported for the saponified fractions were similar between the two products. SFA accounted for  $96.41 \pm 0.08\%$  in mTAC and  $96.47 \pm 0.08\%$  in yTAC, while total UFA represented  $3.59 \pm 0.08\%$  and  $3.53 \pm 0.08\%$ , respectively. MUFA values were  $3.20 \pm 0.08\%$  in both products, and PUFA values were similar for both samples at  $0.39 \pm 0.01\%$ . The n-6 PUFA contents were  $0.23 \pm 0.00\%$  and  $0.23 \pm 0.01\%$ ; the n-3 PUFA contents were  $0.16 \pm 0.01\%$  in both products; and the corresponding n-6/n-3 ratios were  $1.47 \pm 0.04$  and  $1.49 \pm 0.04$ .

**Table 6.** Relative abundance (%) of fatty acids based on normalized LC–MS peak areas after saponification of the polar lipids present in the TAC fractions <sup>1</sup>.

| Empirical Name of Fatty Acid | Formula  | mTAC-Milk (Mean) | SD   | yTAC-Yogurt (Mean) | SD   |
|------------------------------|----------|------------------|------|--------------------|------|
| Caprylic                     | C8:0     | 0.98             | 0.01 | 1.02               | 0.02 |
| Pelargonic                   | C9:0     | 0.07             | 0.01 | 0.08               | 0.01 |
| Capric                       | C10:0    | 1.27             | 0.02 | 1.28               | 0.02 |
| Undecylic                    | (C11:0)  | 0.02             | 0.01 | 0.02               | 0.01 |
| Lauric                       | C12:0    | 5.13             | 0.04 | 5.09               | 0.09 |
| Tridecylic                   | C13:0    | 0.04             | 0.01 | 0.04               | 0.01 |
| Myristic                     | C14:0    | 1.87             | 0.04 | 1.81               | 0.02 |
| Pentadecylic                 | C15:0    | 0.39             | 0.01 | 0.38               | 0.01 |
| Palmitic                     | C16:0    | 33.73            | 0.25 | 33.78              | 0.07 |
| Palmitoleic                  | C16:1 n7 | 0.28             | 0.01 | 0.23               | 0.01 |
| Margaric                     | C17:0    | 1.05             | 0.02 | 1.02               | 0.03 |
| Stearic                      | C18:0    | 51.06            | 0.20 | 51.12              | 0.13 |
| Oleic                        | C18:1 n9 | 2.92             | 0.08 | 2.91               | 0.07 |
| Linoleic                     | C18:2 n6 | 0.23             | 0.01 | 0.23               | 0.01 |
| $\alpha$ -Linoleic           | C18:3 n3 | 0.16             | 0.01 | 0.16               | 0.01 |
| Nonadecylic                  | C19:0    | 0.06             | 0.01 | 0.05               | 0.01 |
| Arachidic                    | C20:0    | 0.74             | 0.03 | 0.79               | 0.01 |
| SFA                          |          | 96.41            | 0.08 | 96.47              | 0.08 |
| MUFA                         |          | 3.20             | 0.08 | 3.14               | 0.07 |
| PUFA                         |          | 0.39             | 0.01 | 0.39               | 0.01 |
| UFA                          |          | 3.59             | 0.08 | 3.53               | 0.08 |
| SFA/UFA                      |          | 26.84            | 0.62 | 27.32              | 0.62 |
| n6PUFA                       |          | 0.23             | 0.01 | 0.23               | 0.01 |
| n3 PUFA                      |          | 0.16             | 0.01 | 0.16               | 0.01 |
| n6/n3                        |          | 1.47             | 0.03 | 1.49               | 0.04 |

<sup>1</sup> Values represent semiquantitative normalized peak-area abundances and should not be interpreted as absolute fatty-acid concentrations. SD = Standard Deviation.

In the non-saponified TAC fractions, the individual fatty acid profiles were closely comparable between mTAC and yTAC. Stearic acid was the most abundant listed free fatty acid (FFA), with values of  $41.23 \pm 0.19\%$  and  $44.17 \pm 0.69\%$ , respectively, followed by palmitic acid at  $28.19 \pm 0.15\%$  and  $28.60 \pm 1.11\%$  (Table 7). Oleic acid the n3  $\alpha$ -linolenic acid showed higher values as FFA in these extracts, at  $8.73 \pm 0.10\%$  and  $11.28 \pm 0.05\%$  in the mTAC, while at  $7.73 \pm 0.47\%$  and  $9.94 \pm 0.64\%$  in the yTAC, respectively. All the other saturated fatty acids (SFA) were at lower concentrations, while for the rest of the unsaturated fatty acids (UFA) low but considerable values were observed for palmitoleic acid and linoleic acid in the FFA of the TAC extracts of both products. At the fatty acid category level of the FFA, the SFA values were  $76.69 \pm 0.01\%$  for mTAC and  $79.43 \pm 1.06\%$  for yTAC. The corresponding UFA values of the FFA were  $23.31 \pm 0.01\%$  and  $20.57 \pm 1.06\%$ ; MUFA values were  $10.41 \pm 0.05\%$  and  $9.23 \pm 0.37\%$ ; and PUFA values were  $12.89 \pm 0.05\%$  and  $11.33 \pm 0.70\%$ , respectively. The n-6 PUFA contents of the FFA were  $1.61 \pm 0.08\%$  and  $1.39 \pm 0.06\%$ , while the n-3 PUFA contents were  $11.28 \pm 0.05\%$  and  $9.94 \pm 0.64\%$ , respectively. The n-6/n-3 ratio of the FFA was  $0.14 \pm 0.01$  for the mTAC extract and similar ( $0.14 \pm 0.01$ ) for the yTAC.

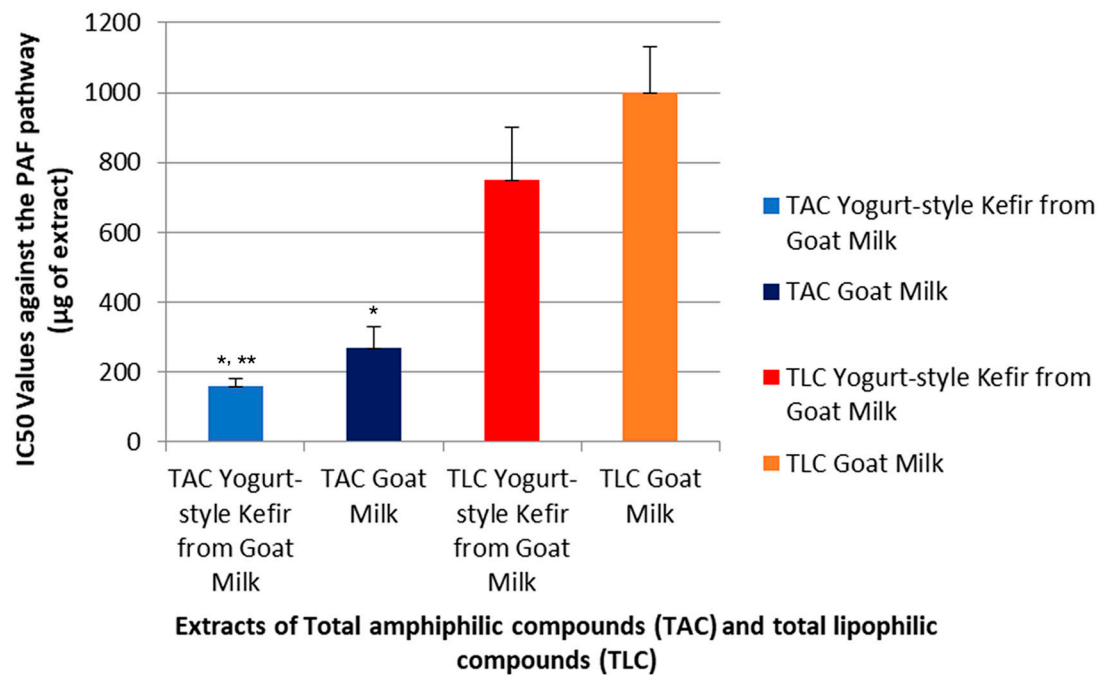
**Table 7.** Relative abundance (%) of fatty acids based on normalized LC–MS peak areas obtained without saponification of TAC fractions (free fatty acids content) <sup>1</sup>.

| Empirical Name of Fatty Acid | Formula  | mTAC-Milk (Mean) | SD   | yTAC-Yogurt (Mean) | SD   |
|------------------------------|----------|------------------|------|--------------------|------|
| Caprylic                     | C8:0     | ND               | ND   | ND                 | ND   |
| Pelargonic                   | C9:0     | 0.75             | 0.01 | 0.79               | 0.05 |
| Capric                       | C10:0    | ND               | ND   | ND                 | ND   |
| Undecylic                    | (C11:0)  | ND               | ND   | ND                 | ND   |
| Lauric                       | C12:0    | ND               | ND   | ND                 | ND   |
| Tridecylic                   | C13:0    | ND               | ND   | ND                 | ND   |
| Myristic                     | C14:0    | 2.40             | 0.04 | 2.24               | 0.15 |
| Pentadecylic                 | C15:0    | 1.94             | 0.07 | 1.71               | 0.07 |
| Palmitic                     | C16:0    | 28.19            | 0.15 | 28.60              | 1.11 |
| Palmitoleic                  | C16:1 n7 | 1.68             | 0.11 | 1.50               | 0.16 |
| Margaric                     | C17:0    | 2.18             | 0.04 | 1.92               | 0.07 |
| Stearic                      | C18:0    | 41.23            | 0.19 | 44.17              | 0.69 |
| Oleic                        | C18:1 n9 | 8.73             | 0.10 | 7.73               | 0.47 |
| Linoleic                     | C18:2 n6 | 1.61             | 0.08 | 1.39               | 0.06 |
| a-Linoleic                   | C18:3 n3 | 11.28            | 0.05 | 9.94               | 0.64 |
| Nonadecylic                  | C19:0    | ND               | ND   | ND                 | ND   |
| Arachidic                    | C20:0    | ND               | ND   | ND                 | ND   |
| SFA                          |          | 76.69            | 0.01 | 79.43              | 1.06 |
| MUFA                         |          | 10.41            | 0.05 | 9.23               | 0.37 |
| PUFA                         |          | 12.89            | 0.05 | 11.33              | 0.70 |
| UFA                          |          | 23.31            | 0.01 | 20.57              | 1.06 |
| SFA/UFA                      |          | 3.29             | 0    | 3.86               | 0.24 |
| n6PUFA                       |          | 1.61             | 0.08 | 1.39               | 0.06 |
| n3 PUFA                      |          | 11.28            | 0.05 | 9.94               | 0.64 |
| n6/n3                        |          | 0.14             | 0.01 | 0.14               | 0.01 |

<sup>1</sup> Values represent semiquantitative normalized peak-area abundances and should not be interpreted as absolute fatty-acid concentrations. SD = Standard Deviation; ND = not detected under the applied assay conditions.

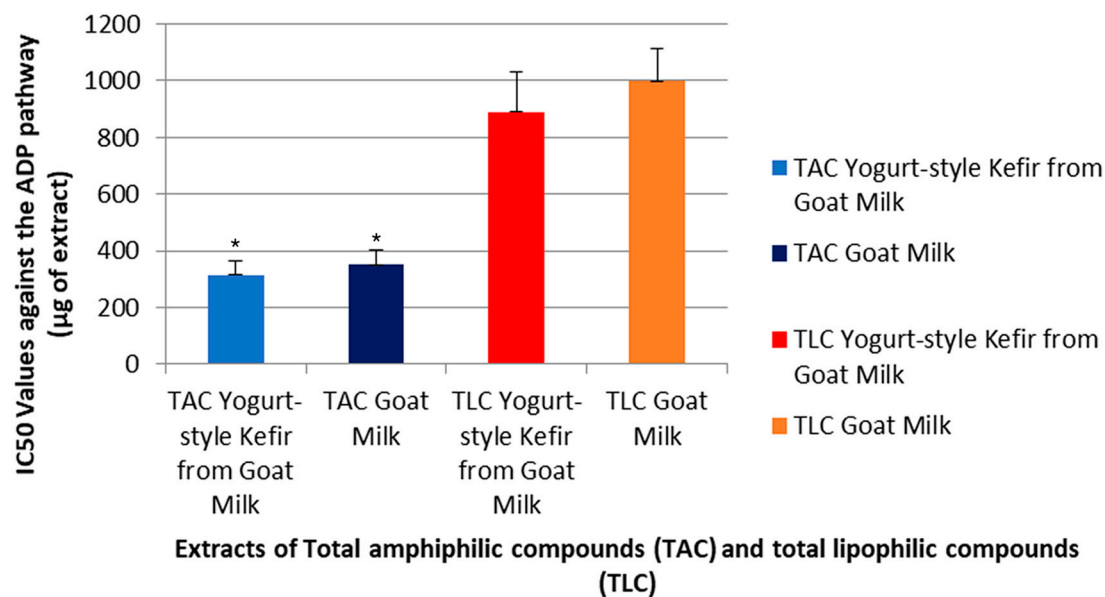
### 3.6. Evaluation of Antithrombotic and Anti-Inflammatory Activity Against ADP and PAF, Respectively, in Human Platelets

Figures 1 and 2 present the inhibitory effects of the TAC and TLC fractions obtained from goat milk and from the kefir-based yogurt-type fermented product against PAF- and ADP-induced platelet aggregation in human platelet-rich plasma (hPRP), respectively. Results are expressed as IC<sub>50</sub> values, defined as µg of dry extract required to inhibit 50% of agonist-induced platelet aggregation in 250 µL hPRP. Values are presented as mean ± SD from 18 individual donor-level determinations per experimental group, comprising three independently prepared extracts evaluated in six different donors per extract. Lower IC<sub>50</sub> values indicate greater inhibitory potency. Against PAF-induced platelet aggregation, both TAC fractions showed significantly lower IC<sub>50</sub> values (158 ± 25 µg of yTAC and 268 ± 60 µg of mTAC, respectively) than their corresponding TLC fractions (749 ± 151 µg of yTLC and 999 ± 133 µg of mTLC, respectively) (ANOVA,  $p < 0.05$ ;  $n = 18$ ). The TAC fraction from the kefir-based yogurt-type product exhibited the lowest IC<sub>50</sub> value among the tested extracts and was significantly more potent than the TAC fraction from goat milk (ANOVA,  $p < 0.05$ ;  $n = 18$ ). The TLC fraction from goat milk showed the highest IC<sub>50</sub> value, followed by the TLC fraction from the kefir-based yogurt-type product.



**Figure 1.** Anti-inflammatory effects against PAF-induced platelet aggregation of TAC and TLC fractions from goat milk and a kefir-based yogurt-type fermented product. Results are expressed as IC<sub>50</sub> values (µg of dry extract required to inhibit 50% of PAF-induced platelet aggregation in 250 µL hPRP), while error bars indicate SD. Lower IC<sub>50</sub> values indicate greater inhibitory potency. (Individual donor-level IC<sub>50</sub> determinations obtained from 3 independent extract preparations × 6 different donors per extract. Individual points represent donor-level IC<sub>50</sub> determinations. For each experimental group, three independently prepared extracts were each evaluated using hPRP from six different healthy donors, yielding 18 individual donor-level IC<sub>50</sub> determinations; 3 extracts × 6 donors per extract. Different donor sets were used across extract preparations; therefore, observations are unpaired across experimental groups). Thus, data are presented as mean ± SD. Statistical comparisons were performed using one-way ANOVA followed by Tukey's HSD post hoc test;  $p < 0.05$  was considered statistically significant. More specifically, \*  $p < 0.05$  for TAC versus the corresponding TLC fraction; \*\*  $p < 0.05$  for yogurt-derived TAC versus goat milk TAC. Abbreviations: TAC, total amphiphilic compounds; TLC, total lipophilic compounds; hPRP, human platelet-rich plasma; IC<sub>50</sub>, half-maximal inhibitory concentration; SD, Standard Deviation.

Against ADP-induced platelet aggregation, the TAC fractions also displayed significantly lower IC<sub>50</sub> values ( $316 \pm 50$  µg of yTAC and  $351 \pm 50$  µg of mTAC, respectively) than their corresponding TLC fractions ( $890 \pm 139$  µg of yTLC and  $998 \pm 114$  µg of mTLC, respectively) (ANOVA,  $p < 0.05$ ;  $n = 18$ ). The IC<sub>50</sub> values of the two TAC fractions were of comparable magnitude, whereas the TLC fractions showed markedly higher values, with the goat milk TLC fraction presenting the highest IC<sub>50</sub> value. Overall, the TAC fractions exhibited greater inhibitory activity than the TLC fractions against both platelet activation pathways. A more pronounced difference between the two dairy matrices was observed for the PAF pathway, where the TAC fraction from the kefir-based yogurt-type product showed the strongest inhibitory response.



**Figure 2.** Antiplatelet effects of TAC and TLC fractions from goat milk and a kefir-based yogurt-type fermented product against ADP-induced platelet aggregation. Results are expressed as IC<sub>50</sub> values (µg of dry extract required to inhibit 50% of agonist-induced platelet aggregation in 250 µL hPRP), while error bars indicate SD. Lower IC<sub>50</sub> values indicate greater inhibitory potency. (individual donor-level IC<sub>50</sub> determinations obtained from 3 independent extract preparations × 6 different donors per extract. Individual points represent donor-level IC<sub>50</sub> determinations. For each experimental group, three independently prepared extracts were each evaluated using hPRP from six different healthy donors, yielding 18 individual donor-level IC<sub>50</sub> determinations; 3 extracts × 6 donors per extract. Different donor sets were used across extract preparations; therefore, observations are unpaired across experimental groups). Thus, data are presented as mean ± SD. Statistical comparisons were performed using one-way ANOVA followed by Tukey's HSD post hoc test;  $p < 0.05$  was considered statistically significant. More specifically, \*  $p < 0.05$  for TAC versus the corresponding TLC fraction. Abbreviations: TAC, total amphiphilic compounds; TLC, total lipophilic compounds; hPRP, human platelet-rich plasma; IC<sub>50</sub>, half-maximal inhibitory concentration; SD, Standard Deviation.

#### 4. Discussion

A comparative characterization of the amphiphilic and lipophilic fractions isolated from commercial liquid goat milk and a commercial kefir-based goat yogurt product demonstrates that biochemical properties and functional activities within caprine dairy matrices are strongly partitioned by polarity. The extraction yields differed numerically between the total amphiphilic content (TAC) and total lipophilic content (TLC) fractions, although the overall differences among groups were not statistically significant. Across both commercial matrices, TLC fractions yielded higher masses than TAC fractions. This distribution reflects the general lipid composition of dairy products, wherein non-polar triacylglycerols constitute the overwhelming bulk substrate, while amphiphilic glycerophospholipids, sphingolipids, and glycolipids represent a smaller membrane-associated pool [12,15]. The lower recovery of TAC extracts is thus consistent with the limited natural abundance of polar lipid species [13,14]. Numerical variations in milk-derived mTAC and kefir-based yTAC should therefore be interpreted as exploratory. Because the evaluation utilized finished commercial retail products originating from non-identical raw milk pools, processing regimes, and starter formulations, it cannot be attributed specifically to fermentation-induced lipolysis or proteolysis [16].

The distribution of carotenoid-associated compounds between the fractions was also influenced by fraction polarity. Total carotenoid content (TCC) varied significantly across matrices and fractions. The goat milk TAC fraction showed the highest median TCC,

whereas carotenoid levels in the kefir-based product were distributed more evenly between yTAC and yTLC. Under the applied biphasic liquid–liquid extraction system, carotenoid-associated species in goat milk were preferentially recovered in the amphiphilic fraction, whereas their partitioning became less distinct in the kefir-based product. The lower TCC recorded in yogurt-derived fractions relative to mTAC may reflect matrix-specific oxidative degradation, processing-induced transformations, or altered phase partitioning [13]. However, in the absence of kinetic tracking during a controlled fermentation process, the mechanisms underlying these differences cannot be established.

Colorimetric Folin–Ciocalteu analysis revealed no detectable total phenolic content (TPC) in unsaponified mTAC and yTAC, whereas low, measurable values were recorded in TLC fractions. The borderline difference in TPC between yTLC and mTLC suggests minor variation in the extractable reducing pool between the commercial matrices. The generally low TPC values across all fractions are consistent with the low abundance of free phenolic compounds reported in caprine dairy matrices, where such compounds may occur at trace levels, interact with proteins, or undergo chemical modifications [18]. Attenuated total reflectance Fourier-transform infrared (ATR–FTIR) spectra provided complementary structural information. While the Folin–Ciocalteu assay measures total reducing capacity, ATR–FTIR spectroscopy identifies characteristic vibrational bands. The presence of broad O–H/N–H stretching bands alongside aromatic C=C features indicates that the absence of detectable TPC in TAC fractions does not preclude the presence of hydroxyl-bearing amphiphilic molecules or trace phenolics below the detection limit of the colorimetric assay [18,21].

Across both commercial matrices, *in vitro* radical-scavenging assays consistently distinguished amphiphilic from lipophilic fractions. In the ABTS assay, TAC extracts exhibited substantially greater radical-scavenging capacity than their corresponding TLC counterparts [19,20]. Median ABTS scavenging capacity did not differ significantly between mTAC and yTAC, indicating broadly comparable antioxidant responses in the two TAC fractions, although yTAC showed lower response variability. The DPPH assay corroborated this trend, with TAC fractions exhibiting markedly higher median TEAC values than TLC fractions [20]. Because colorimetric TPC was undetectable in TAC fractions, this antioxidant activity cannot be assigned solely to free phenolic compounds. Instead, it may result from the combined contribution of a complex amphiphilic mixture comprising polar glycerophospholipids, sphingolipids, carotenoid-associated complexes, free fatty acids, and amphiphilic peptides [19,20]. The infrared spectra are consistent with this chemical heterogeneity, displaying aliphatic C–H stretching bands alongside ester carbonyls and phosphate-related vibrations (P = O, P–O, C–O, and C–O–P) at 1200–1050 cm<sup>−1</sup>, which support the presence and co-extraction of polar lipids and other amphiphilic constituents [18,21].

Relative fatty acid profiling via liquid chromatography–mass spectrometry (LC–MS) allowed comparison of the fatty-acid profiles of the saponified and non-saponified TAC fractions. In saponified fractions, relative abundances—expressed as normalized LC–MS peak area percentages—were dominated by saturated fatty acids (SFAs), with monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs) constituting smaller proportions. Minor numerical variations in individual peak areas, including differences in the relative abundances of palmitic, stearic, and oleic acids, are more appropriately interpreted as compositional differences between the two commercial products rather than as evidence of fermentation-induced changes. Non-saponified TAC fractions similarly displayed palmitic and stearic acids as the predominant free fatty acids.

All TAC extracts showed a low relative n-6/n-3 PUFA ratio, a compositional characteristic that may be relevant to inflammatory pathway modulation [22]. Furthermore, the lower relative recovery of long-chain unsaturated fatty acids in saponified TAC fractions

may reflect the extraction selectivity of the biphasic counter-current system compared with conventional organic-solvent extraction procedures. Crucially, because these LC–MS profiles were generated without internal standards or response-factor corrections, the values represent relative chromatographic abundances rather than absolute quantitative concentrations and cannot be used to characterize intact lipid molecular species [18,21].

Functional bioassays performed in human platelet-rich plasma (hPRP;  $n = 6$  independent donors) showed that TAC fractions possessed significantly greater *ex vivo* inhibitory potency against agonist-induced platelet aggregation than TLC fractions [23–26]. Both mTAC and yTAC displayed significantly lower  $IC_{50}$  values against platelet-activating factor (PAF)- and adenosine diphosphate (ADP)-induced aggregation compared with the corresponding TLC fractions, consistent with previous reports on platelet-active bioactives associated with the caprine milk fat globule membrane [4,5,10,17,24,27]. The two agonists activate distinct biological pathways: PAF is an ether-phospholipid thrombo-inflammatory agonist acting through its specific G-protein-coupled receptor (PAFR), whereas ADP signals through purinergic P2Y receptors [23–26]. The comparable inhibitory potency of mTAC and yTAC against ADP-induced platelet aggregation ( $IC_{50} > 300 \mu g$ ) suggests that the two TAC fractions exert broadly similar effects on this pathway.

Conversely, yTAC exhibited significantly greater inhibitory potency against PAF-induced platelet aggregation, with an  $IC_{50}$  of approximately  $160 \pm 20 \mu g$ , than mTAC. This difference was not explained by the overall fatty-acid class distribution or by colorimetric TPC. It may instead reflect differences in the relative abundance of specific intact polar lipid species, such as phosphatidylcholine, phosphatidylethanolamine, or sphingomyelin, or interactions among free fatty acids, carotenoid-associated compounds, and other amphiphilic constituents present in the two commercial matrices [5,10,17,24,27–39]. Such polar lipid-associated compounds may contribute to the inhibition of PAF-mediated platelet activation through interactions with PAFR or through effects on membrane organization [28–39]. The weak residual aggregation inhibition observed in TLC fractions may similarly reflect the presence of minor co-extracted free fatty acids or other non-polar bioactive constituents [28–31].

Nevertheless, it should also be stressed out that although different independent donors were used for each extract preparation, the platelet-aggregation dataset had a hierarchical experimental structure, comprising three independent extract preparations with six donor-level  $IC_{50}$  determinations per extract. The present statistical analysis was performed on the individual donor-level  $IC_{50}$  values using one-way ANOVA followed by Tukey's HSD post hoc test and did not explicitly model extract-level clustering. This should be considered when interpreting the statistical inference. Future studies incorporating a larger number of independently produced commercial lots could enable hierarchical or mixed-effects statistical approaches to simultaneously account for between-lot/extract and between-donor variability.

Overall, these findings show substantial *in vitro* radical-scavenging activity and *ex vivo* inhibition of PAF- and ADP-induced platelet aggregation by the TAC fractions in hPRP from six independent donors, extending the current understanding of the functional properties of caprine dairy lipid fractions [40–50]. Future studies should employ controlled fermentation experiments with matched raw milk controls, high-resolution targeted LC–MS/MS lipidomics to identify intact polar lipid species, simulated gastrointestinal digestion models to assess bioaccessibility, and validated *in vivo* models to evaluate the physiological relevance of the observed activities.

## 5. Conclusions

This study provides a comparative assessment of the chemical, structural, and functional properties of amphiphilic (TAC) and lipophilic (TLC) fractions isolated from commercial liquid goat milk and a commercial kefir-based goat yogurt product. Across both commercial matrices, TAC fractions exhibited greater in vitro radical-scavenging activity in the DPPH and ABTS assays and significantly stronger ex vivo inhibition of PAF- and ADP-induced platelet aggregation than the corresponding TLC fractions. ATR–FTIR analysis revealed functional groups consistent with a chemically heterogeneous amphiphilic matrix, while LC–MS profiling characterized differences in the relative fatty-acid composition of the TAC fractions.

The greater inhibitory potency of TAC fractions against PAF-induced platelet aggregation, particularly that of the kefir-based product, supports the potential functional relevance of polar lipid-associated constituents in caprine dairy matrices. However, because the two commercial products originated from distinct raw materials, processing conditions, and starter formulations, differences between them should be considered exploratory and cannot be attributed specifically to fermentation. Furthermore, the observed in vitro radical-scavenging and ex vivo platelet-inhibitory activities should not be extrapolated to systemic anti-inflammatory, antithrombotic, or cardioprotective effects. Further studies using controlled fermentation designs, targeted high-resolution lipidomics, gastrointestinal digestion models, and appropriate in vivo approaches are required to identify the compounds responsible for these activities and establish their physiological relevance.

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**Institutional Review Board Statement:** The platelet-aggregation experiments involving blood samples from healthy volunteers were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of DEMOCRITUS UNIVERSITY OF THRACE (protocol codes ΔΠΘ/ΕΗΔΕ/7690/70; approval date: 27 September 2024 and ΔΠΘ/ΕΗΔΕ/73216/640; approval date: 24 June 2026). The experimental study extended across the periods covered by these successive approvals, and all blood collection and platelet experiments were performed under a valid ethical approval in force at the time of experimentation.

**Informed Consent Statement:** Informed consent was obtained from all healthy blood donors involved in the study.

**Data Availability Statement:** The raw data supporting the conclusions of this article will be made available by the PI of the project and the corresponding author (A.T.) upon request due to the need for specific software, hardware and analytical equipment to handle and visualize raw data of the full range of ATR-FTIR spectra analysis, LC-MS analysis and platelet aggregometry analysis.

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