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*Proceedings*

# The *ex vivo* and *in vitro* antithrombotic properties of fermented Irish ovine yogurt drink

**†Sakshi Hans [1], Harishkumar Rajendran [1], Katie Shiels [3], Sushanta Kumar Saha [3], Alexandros Tsoupras [3], Ronan Lordan [4,*] and Ioannis Zabetakis [1,*]**

[1] Department of Biological Sciences, University of Limerick, **(**sakshi.hans@ul.ie**),** (harishkumar.rajendran@ucd.ie), **(ioannis.zabetakis@ul.ie)**

[2] Shannon Applied Biotechnology Centre, Technological University of the Shannon, Moylish Park, Limerick, Ireland, (katie.shiels@tus.ie), **(**sushanta.saha@tus.ie**).**

[3] Department of Chemistry, School of Sciences, International Hellenic University, Saint Luke, GR65404, Kavala, Greece, **(**atsoupras@gmail.com**).**

[4] Institute for Translational Medicine and Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, 19104, USA, **(ronan.lordan@pennmedicine.upenn.edu).**

* Correspondence: ronan.lordan@pennmedicine.upenn.edu; ioannis.zabetakis@ul.ie

**Abstract:** Platelet function is closely linked with cardiovascular health. Functional foods such as yogurt and oily fish are enriched in bioactive polar lipids which can reduce platelet activation and incidence of cardiovascular disease. This project thus investigated the effect of fermented ovine yogurt on platelet sensitivity in human plasma. Overall, in vitro studies established inhibitory effect on platelet aggregation by yogurt lipid fractions. Results from dietary intervention showed that yogurt intake reduces platelet activation against thrombin pathway, compared to placebo. Larger scale studies are required to robustly establish the effect of yogurt intake on platelet sensitivity, following this interim analysis.

**Keywords:** yogurt; polar lipids; platelet aggregation; thrombin-activator receptor pep-tide-6 (TRAP-6); nutraceuticals; antithrombotic; functional foods

## 1. Introduction

While a measured immune response is a necessary protective physiological reaction in response to tissue damage or pathogenic insults, unresolved inflammation is implicated in the pathology of many chronic diseases (Neher et al., 2011). The initiation of cardiovascular diseases (CVD) and other conditions have been linked to low-grade systemic inflammation (Furman et al., 2019, Zotova et al., 2010). Platelets are important effectors of these immune responses by mechanisms such as recruitment of neutrophils to the site of inflammation, and by the release of proinflammatory factors and chemokines that further activate leukocytes and intensify the immune response (Stokes and Granger, 2012, Gros et al., 2014). Platelet activity can be modulated by stimuli such as platelet agonists which contribute to platelet aggregation, or platelet inhibitors, which deactivate platelets and discourage formation of thromboses (Willoughby, 2002). Thrombin receptor activator peptide-6 (TRAP-6) is a peptide fragment that selectively activates the thrombin receptor protease-activated receptor 1 (PAR-1), thus inducing platelet activation similar to thrombin. Thrombin is a serine protease that is a key enzyme in the coagulatory pathway. It signals by binding to the protease-activated receptors (PARs) which are a family of transmembrane peptide receptors (Coughlin, 2000, Reiter et al., 2003). Platelet-activating factor (PAF) is a phospholipid mediator of inflammation that also induces platelet activation and aggregation. PAF functions by binding to the PAF receptor (PAF-R) a G-protein

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coupled receptor (GPCR) located on cells involved in immune function (Yost et al., 2010, Harishkumar et al., 2022).

Consumption of foods or nutraceuticals rich in bioactive lipids (dietary polar lipids) that target platelet signalling pathways such as thrombin and PAF may be beneficial against inflammatory diseases (Harishkumar et al., 2022). However, research is still at a preliminary stage to date, which necessitates the need for further study. Numerous studies have been conducted to investigate the cardioprotective properties of polar lipids such as marine and dairy-derived lipids *in vitro* (Tsoupras et al., 2018, Lordan et al., 2019b, Glenn-Davi et al., 2022, Lordan et al., 2019a). These dietary polar lipids appear to inhibit platelet aggregation induced by agonists such as PAF and TRAP-6.

Ovine or sheep's milk has been noted for its many potential health benefits besides its rich nutritional content, such as its anti-inflammatory, antiplatelet, and antimicrobial properties (Balthazar et al., 2017, Mohapatra et al., 2019). Fermentation of ovine milk has been shown to further enhance its antithrombotic properties by altering the fatty acid profile of monounsaturated fatty acids (MUFA) (Lordan et al., 2019b, Glenn-Davi et al., 2022).

The aim of this study was to assess the antiplatelet properties of ovine yogurt drink (YD) enriched in polar lipids using platelet aggregometry *in vitro* and to assess the effect of its long-term consumption on platelet sensitivity in a randomized controlled trial.

## 2. Materials and Methods

### *2.1. Materials and Instrumentation*

All consumables and solvents were purchased from Fisher Scientific Ltd. 22G safety needles and evacuated S-monovettes were purchased from Sarstedt Ltd. Platelet aggregometry consumables were purchased from Labmedics LLP. Other reagents and chemicals were purchased from Sigma Aldrich. Platelet aggregation testing was performed on a Chronolog-490 two channel turbidimetric platelet aggregometer and its accompanying AGGRO/LINK software.

### *2.2. Production of the yogurt drink and placebo yogurt drink*

Ewe's whole milk was obtained from Velvet Cloud Ltd. (Claremorris, Co Mayo, Ireland), from Friesland and Lacaune breed of dairy ewes. The percentage of fat in raw milk was approximately 7%. The milk was pasteurised on site by heating to 91 °C for 15 s, and then cooled to 42 °C before being packaged and refrigerated (4 °C ± 1 °C). F.

After pasteurization, the milk was divided into two smaller batches and refrigerated. From one of these batches, skimmed milk was produced by double centrifugation; initially by centrifugation at 6900 x g for 30 minutes at 40°C, and afterwards at 7440 x g for 30 min also at 40°C. After both centrifugations, the settled layer of fat was removed to produce skimmed milk. Both milk batches were then inoculated with commercially available YO-MIX 205 LYO 250 DCU starter culture (Danisco France SAS, France; 5 mg/100 mL whole milk) to induce fermentation at a temperature of 40.5 °C for a period of 7h. The milk batches were placed in a water bath to ferment and reach a pH = 4.6 and desired consistency (thick and creamy). The cultures contained the following probiotics- *Streptococcus thermophilus*, *Lactococcus delbrueckii* subsp. *Bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium lactis.* After fermentation the milk was then poured into 250 ml bottles and sealed.

### *2.3. Extraction of lipids and determination of nutrient content*

Total lipids (TL) of yogurt drink (YD) and placebo drink (PD) were extracted from 100 g of sample according to the Bligh and Dyer technique [21]. Total neutral lipids (TNL) and total polar lipids (TPL) were further isolated from TL by counter-current distribution method [22]. Samples were stored under nitrogen at -20 °C.

*2.4. In vitro assessment of antithrombotic properties of lipid fractions against TRAP-6 and PAF-induced platelet aggregation*

Platelet inhibition was assessed for each lipid fraction using platelet aggregometry as detailed in Table 2. Various subclasses of TPL were isolated using preparative thin-layer chromatography (TLC) and the IC50 of these was determined similarly (Table 3). The half-maximal inhibitory concentration ($IC_{50}$) was expressed as the mean mass of lipid fraction (μg) in the aggregometer cuvette ± standard deviation (SD). All experiments were performed in triplicate.

*2.5. Dietary intervention study of Irish ovine yogurt drink*

The dietary intervention was designed as a randomized double-blind crossover study with the control group provided a placebo drink containing significantly lower amounts of polar lipids (n = 18) produced as described in section 2.2. Two study phases were organized with equally divided groups and each phase of trial was conducted for 28 days. Figure 1 is a schematic representation of the study design.

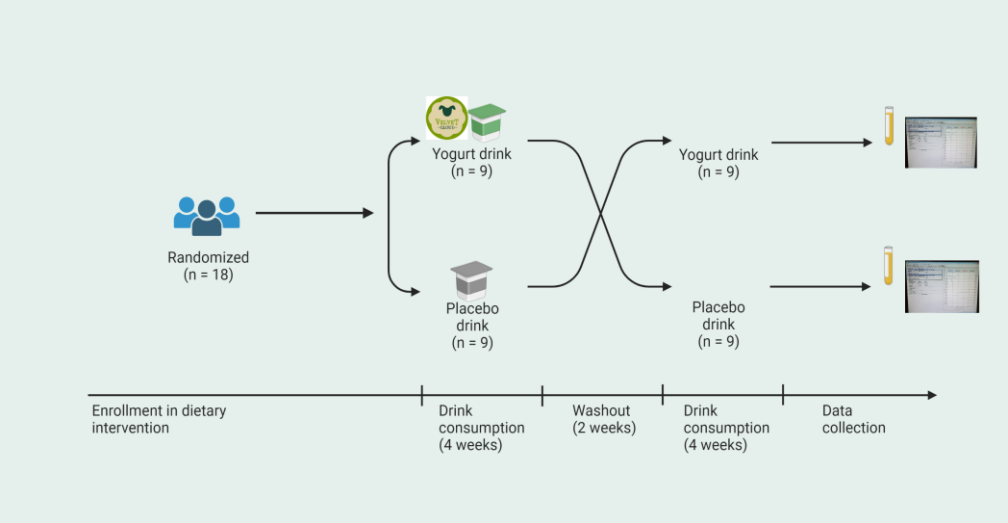


**Figure 1.** Schematic representation of the design of the crossover dietary intervention study. 18 participants were recruited in the randomized study with equal numbers assigned 250 gm of either the yogurt drink or placebo drink. Blood sampling was performed at four timepoints with a washout period of two weeks prior to the crossover phase.

## 3. Results

*3.1. Yield of lipid fractions isolated from ovine yogurt drink*

A side-by-side comparison of the ovine yogurt drink and placebo drink with respect to their nutritional content is given in Table 1. The yogurt drink was significantly higher in fat content compared to the placebo (prepared from skimmed milk).

**Table 1.** Yield of the lipid fractions (TL, total lipid; PL, polar lipids; NL, neutral lipids) from raw sheep's milk, skimmed milk-derived placebo drink and full fat yogurt drink (g/100mL). Data is presented as mean ± SD.

| Samples (100 mL) | Total lipids (TL) in grams | Polar lipids (PL) in gm | Neutral lipids (NL) in gm |
|---|---|---|---|
| Raw ovine milk | 9.96 ± 0.30 [a] | 0.16 ± 0.01 [a] | 8.24 ± 0.23 [a] |
| Yogurt drink | 8.17 ± 0.22 [a] | 0.15 ± 0.004 [a] | 7.75 ± 0.19 [a] |
| Skimmed milk-derived yogurt drink (Placebo) | 0.36 ± 0.01 [b] | 0.06 ± 0.002 [b] | 0.29 ± 0.01 [b] |

[a,b,] Different superscripts in the same column indicate significant differences among different lipid extracts within the same lipid class ($p < 0.05$) when means are compared using a Tukey's HSD

multiple comparison test. Polar lipids (PL) are amphiphilic and possess a hydrophilic head and hydrophobic tail. Neutral lipids (NL) have a hydrophobic structure and lack charge.

*3.2. In vitro assessment of antithrombotic properties of lipid fractions against TRAP-6 and PAF-induced platelet aggregation*

Lipid fractions (TL, TNL, and TPL) were extracted and assessed for bioactivity against PAF and TRAP-6 (**Table 2**.).

**Table 2.** Inhibitory effects ($IC_{50}$ values) of polar lipids (PL), neutral lipids (NL), and total lipids (TL) isolated from the ovine yogurt drink against human platelet aggregation induced by PAF and TRAP-6 *in vitro*. Data is expressed as mean ± SD.

| | | IC50 against TRAP-6 (μg) | IC50 against PAF (μg) |
|---|---|---|---|
| Yogurt drink | YPL | 312.95 ± 15.04 | 156.72 ± 5.88 |
| | YNL | 259.41 ± 13.56 | 729.15 ± 35.079 |
| | YTL | 209.57 ± 10.11 | 248.23 ± 11.39 |
| Placebo (skimmed milk) | YPL | 45.8 ± 2.05 | 44.0 ± 1.98 |
| | YNL | 47.37 ± 2.14 | 47.20 ± 2.13 |
| | YTL | 59.16 ± 3.13 | 53.84 ± 2.85 |

[a,b,c,d,e] Different superscripts in the same column indicate significant differences among different lipid extracts within the same lipid class ($p < 0.05$) when means are compared using a Tukey's HSD multiple comparison test.

*3.3. In vitro assessment of antithrombotic properties of each PL subclass towards TRAP-6 and PAF-induced platelet aggregation in PRP, expressed as mean ± SD.*

**Table 3** depicts the in vitro inhibition of the subclasses of yogurt TPL in response to PAF and TRAP-6-induced platelet aggregation, represented as $IC_{50}$ values for each TLC fraction. The lipid fractions corresponding to PC (phosphatidylcholine) had the lowest $IC_{50}$ values against PAF and TRAP and consequently the highest biological activity, closely followed by the PE fraction against PAF.

**Table 3.** Antiplatelet activity of each PL subclass towards PAF-induced platelet aggregation in PRP, expressed as mean ± SD.

| Yogurt drink polar lipid fractions | | $EC_{50}$ (-) or $IC_{50}$ against PAF (μg) | $IC_{50}$ against TRAP-6 (μg) |
|---|---|---|---|
| 1 | LPC | -48.1 ± 3.9 | 348.6 ± 7.9 |
| 2 | SM | 1120 ± 54 | 250.6 ± 8.3 |
| 3 | PC | 169.5 ± 8.3 | 205.3 ± 8.9 |
| 4 | CL | ND | 208.0 ± 10.8 |
| 5 | PE | 196.7 ± 6.4 | 592.1 ± 8.8 |

**Abbreviations:** CL, cardiolipin; LPC, lyso-phosphatidylcholine; ND, not determined; PAF, platelet-activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; TRAP-6, thrombin receptor activator peptide 6.

*3.4. Assessment of the impact of dietary yogurt intake on platelet sensitivity against TRAP-6*

**Table 4** depicts the effect of yogurt consumption on TRAP-6 induced platelet activity, compared to placebo, at different timepoints during the study. Overall, a trend of increased EC50 in the YD cohort was observed indicating reduced platelet activation status, although not statistically significant.

**Table 4.** The effect of yogurt (YD) consumption on platelet activation by TRAP-6, expressed as the mean ΔEC50 at different timepoints.

| Treatment group | ΔEC50 Day 0-28 | ΔEC50 Day 42-70 | Combined ΔEC50 |
|---|---|---|---|
| **YD** | 17.22 ± 32.2 | 10.22 ± 33.35 | 13.72 ± 31.1 |
| **PD** | 7.45 ± 27.5 | -5.97 ± 19.95 | 0.742 ± 23.6 |

## 4. Discussion

Research has shown that fermented ovine dairy has potent antiplatelet effects against the thrombin pathway of platelet activation, in vitro in human PRP. [37]. The effect of daily intake of yogurt on the PAF pathway of platelet activation has also been demonstrated as a dietary intervention study model, although no intervention studies so far have demonstrated the ex vivo effect of yogurt on platelet activation through the thrombin pathway [33]. Due to funding constraints the present study did not investigate the effect of non-fermented ovine milk on TRAP-6 platelet activation pathway. However, in vitro data suggests there is less potent activity of non-fermented milk vs fermented yogurt. Thus, the present dietary intervention study sought to determine the effect of consumption over 28 days of an ovine yogurt drink (YD) enriched with polar lipids on baseline platelet reactivity, through the thrombin pathway of activation. In vitro analyses found that yogurt lipids inhibit platelet activation through both PAF and thrombin pathways. While we observed a clear trend of yogurt intake reducing platelet activation through thrombin pathway in human volunteers, studies on a larger scale are required to establish impact of yogurt lipids more robustly on platelet activity.

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