Heliyon 8 (2022) e09025

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Entourage effect for phenolic compounds on production and metabolism of mammary epithelial cells

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ARTICLE INFO

Keywords:
Mammary gland
Milk
Triglyceride
Casein
Polyphenol

ABSTRACT

Primary culture of mammary epithelial cells (MEC) was exposed to ethyl-acetate, chloroform and hexane extracts of *Pistacia lentiscus* (lentisk). The hexane extract contained mainly ethyl gallate whereas the chloroform extract contained mainly ethyl-gallate with smaller amount of gallic acid, and the ethyl-acetate extract contained mainly rutin, gallic acid and myricetin. Ethyl acetate extract increased secretion of protein and fat and improved mitochondrial activity. The enhancing effect on protein production was attributed to myricetin, one of the polyphenols in the ethyl-acetate extract whereas gallic acid did not affect protein production or secretion. Interestingly, exposure to the isolated polyphenols did not improve mitochondrial productivity and activity as effectively as exposure to the complete plant extract. The results indicated that polyphenols improve production of milk constituents by MEC, through different modes of action for different polyphenols suggesting an additive or even synergistic effect on production traits of mammary cells.

1. Introduction

The mammary epithelial cells (MEC) in the mammary gland are responsible for the synthesis and secretion of milk constituents; they are characterized by high metabolic rates and high reactive oxygen species (ROS) levels (Bernabucci et al., 2005). ROS are oxidizing intermediates produced by cellular metabolism involving oxygen consumption. A certain level of ROS is required for normal cellular activity, and they are utilized by mammalian cells as signal transducers for growth by stimulating cell division (Finkel, 1998). However, excessive production or accumulation of ROS is detrimental to cells, as they contribute to cellular aging (Sastre et al., 2000), mutagenesis (Takabe et al., 2001), DNA breakage (Takabe et al., 2001), and lipid peroxidation (Takabe et al., 2001). In addition, ROS levels are elevated in response to MEC inflammation (Deng et al., 2017) and can induce apoptosis (Thomas et al., 2011), whereas inhibition of ROS production under inflammatory conditions leads to suppression of inflammatory cytokine expression (Wang et al., 2018). Taken together, these findings suggest that the high metabolic requirements for production in MEC are accompanied by excessive production and accumulation of ROS, which in turn may impair the cells' redox status.

To mitigate the effect of ROS, mammalian cells utilize endogenous and exogenous antioxidants. The endogenous system involves enzymes such as catalase and superoxide dismutase, whereas the exogenous system utilizes food-borne antioxidants such as vitamin C and alpha tocopherol (Cheeseman and Slater, 1993), and polyphenols, in particular flavonoids (Chen et al., 2014). Plants with high secondary metabolite contents are a natural source for exogenous antioxidants such as polyphenols and flavonoids, known for their antioxidant and anti-inflammatory effects (Vanzani et al., 2011).

The leaves of *Pistacia lentiscus* (lentisk) contain a high concentration of phenolic compounds, such as gallic acid, myricetin and rutin (Azaizeh et al., 2013; Rodríguez-Pérez et al., 2013), which can reach up to 20% of the leaf mass on a dry matter basis (Azaizeh et al., 2015; Glasser et al., 2009). For centuries, traditional human and veterinary medicine has used extracts of *P. lentiscus* to treat cough, and eczema (Palevitch et al.,

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https://doi.org/10.1016/j.heliyon.2022.e09025
Received 6 June 2021; Received in revised form 26 October 2021; Accepted 24 February 2022

2000), and against endo- and ectoparasites (Landau et al., 2014). Aqueous extract of *P. lentiscus* leaves has been suggested as an effective treatment against hepatic jaundice in rats (Janakat and Al-Merie, 2002), and to relieve symptoms of Crohn's disease in humans (Kaliora et al., 2007). In vitro, aqueous extraction of *P. lentiscus* leaves inhibited lipid peroxidation in rat and human adrenal cell lines (Ljubuncic et al., 2005), and scavenged free radicals in a cell-free system (Atmani et al., 2009; Baratto et al., 2003).

In vivo, when given at suitable levels, dietary polyphenols increase milk production and quality traits (O'Callaghan et al., 2016; Argov-Argaman et al., 2016). These effects are commonly attributed to the activity of polyphenols in the gastrointestinal tract (Patra and Saxena, 2011), and have been suggested to result from the duodenal flow of amino acids (Aerts et al., 1999) or from a change in the rumen microbiome (Henke et al., 2017). However, it seems that polyphenol activity is not limited to the gut, because they can be absorbed into the bloodstream and even transported to milk (O'connell and Fox, 2001). Considering the anatomical structure of the mammary gland and the presence of a blood–milk barrier, mammary gland cells are directly exposed to phenolic compounds originating from feed, but the direct effect of these compounds on MEC production traits has yet to be determined.

The effect of polyphenols on the mammary gland has been demonstrated under induced inflammation, and a positive effect of polyphenols during pathological conditions was demonstrated in a mouse model of induced mastitis (Su et al., 2019), in cultured bovine mammary gland cell lines (Jin et al., 2016), and even in goats fed on phenolic glucoside-rich willow (*Salix acmophylla*) (Muklada et al., 2020). However, it is not clear whether polyphenols affect milk production directly in MEC. Moreover, whereas for the most part, these studies demonstrated the effect of a single isolated polyphenol compound, plant extracts consist of a variety of polyphenols, which may exert different effects on cellular processes. In fact, a combination of polyphenols may have a synergistic or additive effect on various physiological and cellular processes. For example, exposure to pure pomegranate juice decreased the viability of human oral, prostate, and colon tumor cells more than the main polyphenols in pomegranate, punicalagin and ellagic acid, administered as isolated compounds (Seeram et al., 2005). Additional evidence for a synergistic effect of plant secondary metabolites has been described for cannaboids, terpenes and flavonoids from cannabis plants, a phenomenon often referred to as "entourage effect" (Russo, 2011). Whether the “entourage effect” also applies to production traits, in particular in MEC, has yet to be studied.

Here, we examined the effect of lentisk extracts and of some of their individual phenolic components on the milk secreted by bovine primary cultured cells. We used a reductionist approach to assign specific roles to individual polyphenols in cellular energy-producing mechanisms and antioxidant activity, and compared these to the whole plant extract, which consists of an array of polyphenols.

2. Results

The average yield of the ethanolic crude extract from the *P. lentiscus* leaves was 30% (g/g) and was similar to those obtained in previous study (Azaizeh et al., 2015). For further fractionation, obtained from 20 g of the crude ethanol extract of *P. lentiscus*, the highest yield was obtained from ethyl-acetate extraction (3.78%), which was 6 times higher than chloroform (0.62%) or hexane fractions (0.56%). The phenolic compounds remaining (95.04%) after fractionation were discarded because they showed a very low activity on the MEC.

Total phenols, flavonoids and tannins contents ranged ethyl acetate fraction > chloroform fraction > hexane fraction (Table 1). The fractions differed in the concentration and composition of the phenolic compounds (Table 2). The ethyl acetate fraction showed a wide variety of phenolics, while in the fractionation process using hexane and chloroform, the number of phenolics was reduced (Figure 1 and Table 2). In the ethyl acetate fraction, seven compounds were identified:

Table 1. Total phenol, flavonoids and tannins concentration in the hexane, chloroform, and ethyl-acetate fractions.

Plant fraction	Total phenolic content (mg Gallic acid/g extract)	Total flavonoids content (mg Quercetin/g extract)	Tannins content (mg tannic acid/g extract)
Hexane fraction	0.78 ± 0.50	0.10 ± 0.73	0.20 ± 0.92
Chloroform fraction	23.7 ± 9.1	32.0 ± 14.6	45.5 ± 18.2
Ethyl acetate fraction	44.6 ± 0.7	66.5 ± 1.1	87.5 ± 1.4

Table 2. The concentration (mg/g) of phenolic compounds detected in the hexane, chloroform, and ethyl-acetate fractions using HPLC analysis.

Fraction	Phenol	Concentration (mg/g)
Hexane fraction	Ethyl gallate	0.007
Chloroform fraction	Gallic acid	0.008
	3,4 Hydroxy benzoic acid	0.011
	Ethyl gallate	0.381
Ethyl acetate fraction	Gallic acid	0.127
	3,4 Hydroxy benzoic acid	0.007
	Catechin	0.042
	Syringic acid	0.056
	Ethyl gallate	0.078
	Rutin derivatives	0.337
	Myricetin derivatives	0.075

gallic acid; 3,4 hydroxy benzoic acid; catechin; syringic acid; ethyl gallate; Rutin derivatives; and myricetin derivatives (Table 2). When antioxidant activity was expressed as EC50 value, the ethyl acetate fraction exhibited a higher antioxidant activity than the chloroform fraction. The antioxidant capacity of the hexane fraction was under the detection level (Table 3).

2.1. Production traits in MEC exposed to different fractions of P. lentiscus

Exposure to hexane fraction at 1 and 10 ppm, but not lower concentrations, was toxic to MEC, as evidenced by cell nucleus amorphism and low cell counts (Figure 2). The secretion of triglycerides, lactose, casein and whey proteins by MEC did not change upon exposure to 0.01 and 0.1 ppm hexane fraction compared to controls ($P = 0.92$, $P = 0.88$, $P = 0.77$, $P = 0.07$, respectively, Figure 3). A similar result was noted following exposure to 1 ppm chloroform fraction, whereas 10 ppm chloroform fraction tended to increase secretion of whey protein ($P = 0.05$) and significantly ($P < 0.05$) increased secretion of triglycerides, lactose and casein by 111%, 25% and 17%, respectively compared with control (Figure 3). Exposure to 10 ppm ethyl-acetate fraction significantly increased the secretion of lactose, casein and whey protein by 30% ($P < 0.05$, $P < 0.05$, $P < 0.05$, respectively, Figure 3), whereas exposure to 1 ppm ethyl-acetate fraction did not change the concentration of the tested metabolites compared to control (Figure 3).

2.2. Lipid droplet size in MEC exposed to different fractions of P. lentiscus

Intracellular lipid droplets in MEC were greater 19% and 70% in diameter, compared with the controls, following exposure to 1 and 10 ppm of the ethyl-acetate fraction ($P < 0.05$ and $P < 0.05$, respectively, Figure 4B). This effect was not noted following exposure chloroform fraction ($P = 0.86$) while a tendency was found when exposed to hexane extract ($P = 0.08$, Figure 4B).

Lipid droplet size is a proxy for the cellular content of triglyceride, although some other mechanisms can control the intracellular droplet

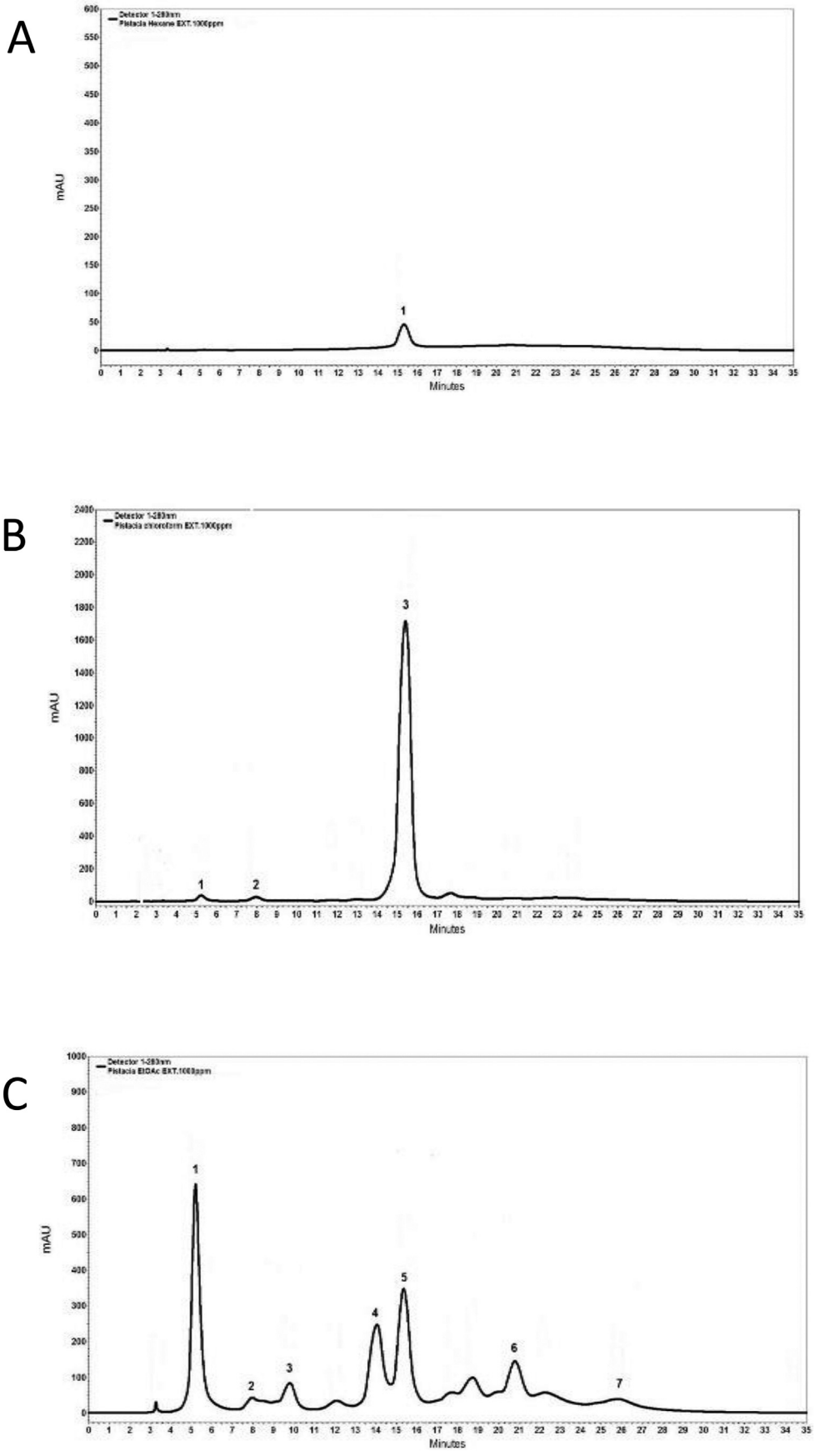

Figure 1. The identification and quantification of three *Pistacia lentiscus* fractions (hexane, chloroform, ethyl-acetate) obtained from 1000 ppm of the different fractions analyzed using HPLC (A) Identification of the phenols in hexane fraction: 1- Ethyl gallate (B) Identification of the phenols in the chloroform fraction: 1- Gallic acid; 2–3,4 Hydroxy benzoic acid; 3- Ethyl gallate (C) Identification of the phenols in Ethyl-acetate fraction: 1- Gallic acid; 2–3,4 Hydroxy benzoic acid; 3- Catechin; 4- Syringic acid; 5- Ethyl gallate; 6- Rutin derivatives; 7- Myricetin derivatives.

Table 3. EC_{50} values of the different fractions.

Fraction	DPPH EC_{50} (mg/l)[1]
Hexane fraction	n.d[2]
Chloroform fraction	824.5 ± 0.009
Ethyl acetate fraction	45.67 ± 0.03

[1] EC_{50} value defined as the concentration required to neutralize a 50% of the DPPH radicals, calculated by linear regression of % if neutralizing effect plotted against extract concentration.
[2] n.d Low activity under detection level.

size like the stability of the membrane (Argov-Argaman, 2019). Therefore, we determined the triglyceride and phospholipid (PL) content and composition in MEC exposed to hexane, chloroform, and ethyl acetate fractions. We found that exposure to 10 ppm ethyl-acetate fraction increased intracellular triglyceride content by 182% compared with control ($P < 0.01$, Figure 4C), but no effect was noted for phospholipid content (Figure 5A). However, the composition of PL was modified: Exposure to 10 ppm ethyl acetate fraction increased Phosphatidylethanolamine (PE) 214% ($P < 0.1$, Figure 5B), Phosphatidylserine (PS) tended to be higher ($P = 0.09$, Figure 5B), but Phosphatidylcholine (PC) content tended to be 15% lower than in the controls ($P = 0.1$, Figure 5C). The ratio of PC to PE tended to be lower following exposure to 10ppm ethyl-acetate compared with the control group (40%, $P < 0.10$, Figure 5D).

2.3. Mitochondria indices in MEC exposed to different fractions of *P. lentiscus*

No difference in mitochondria content was found between ethyl acetate treatment and control groups (Figure 6B). In order to assess

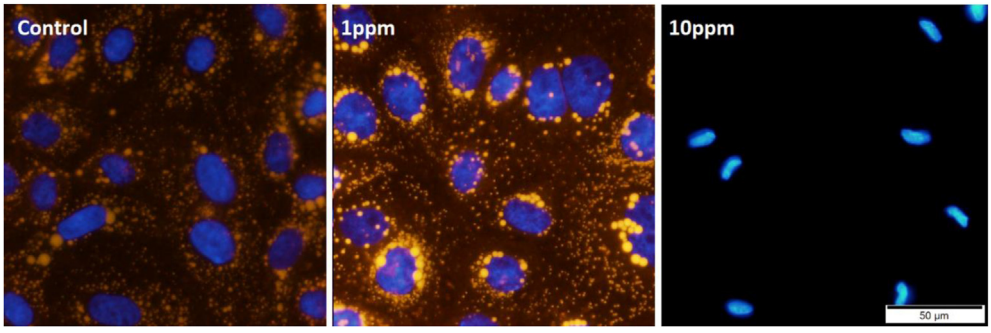

Figure 2. Intracellular manufacturing properties of bovine mammary epithelial cells after exposure to 24 h of hexane fraction obtained from *Pistacia lentiscus* crude ethanol extract in culture medium. After cultivating mammary epithelial cells with *Pistacia lentiscus* hexane fraction for 24 h, intracellular lipid droplets stained in Nile red. Representative images showing the intracellular phenotype after lipid droplets fluorescent staining (Dapi – Blue, Nile red – Yellow).

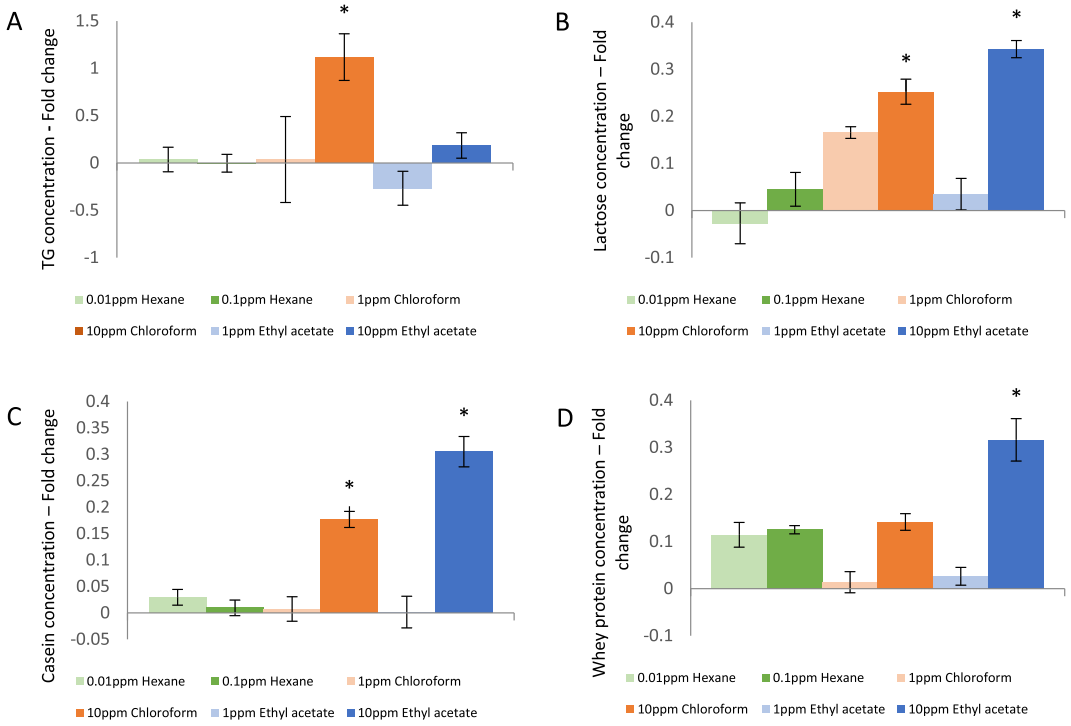

Figure 3. Extracellular manufacturing properties of bovine mammary epithelial cells after exposure to 24 h of hexane, chloroform or ethyl acetate fractions obtained from *Pistacia lentiscus* crude ethanol extract in culture medium. After cultivating mammary epithelial cells with *Pistacia lentiscus* hexane, chloroform and ethyl acetate fractions for 24 h, the medium was collected and taken to HPLC to analysis of triglyceride, protein and lactose. (A) Triglyceride concentration. (B) Lactose concentration. (C) Casein protein concentration. (D) Whey protein concentration. Differences in triglycerides, lactose casein and whey protein content between treatment are expressed in fold of change compared with control. Data obtained from two independent experiments with 4 replicates for each treatment (n = 4). All data are presented as mean ± SD. * indicate significant differences between treatment to control group ($P < 0.05$).

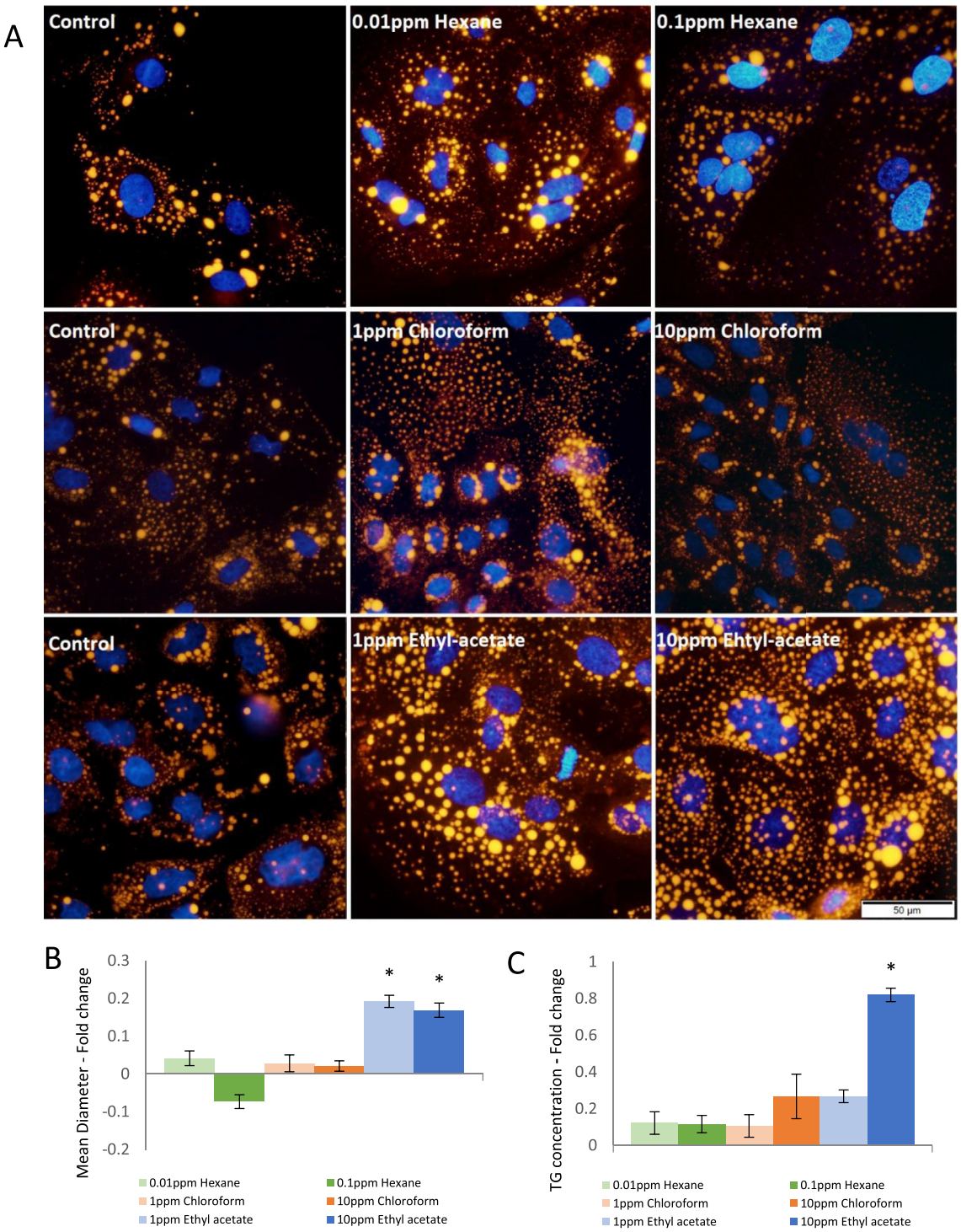

Figure 4. Intracellular manufacturing properties of bovine mammary epithelial cells after exposure to 24 h of hexane, chloroform or ethyl acetate fractions obtained from *Pistacia lentiscus* crude ethanol extract in culture medium. After cultivating mammary epithelial cells with *Pistacia lentiscus* hexane, chloroform and ethyl acetate fraction for 24 h, intracellular lipid droplets stained in Nile red, the cells were taken to HPLC to analysis of triglyceride. (A) Representative images showing the intracellular phenotype after lipid droplets fluorescent staining (Dapi – Blue, Nile red – Yellow). (B) After staining lipid droplets, the droplets were counted, and the mean diameter was measured. (C) Triglyceride concentration. Differences in lipid droplets diameter and triglycerides content between treatment are expressed in fold of change compared with control. Data obtained from two independent experiments with 4 replicates for each treatment (n = 4). All data are presented as mean ± SD. * Indicate significant differences between treatment to control group ($P < 0.05$).

whether polyphenols extracts changed the mitochondria ability to maintain membrane potential during oxidative stress, membrane potential of the mitochondria after exposure to hydrogen peroxide was assessed. We found that MEC pre-exposed to 10 ppm ethyl-acetate fraction for 24 h before the oxidation challenge had a better mitochondria membrane potential after exposure to 0.5mM H_2O_2 for 2 h, compared to control. Cells treated with 10 ppm ethyl-acetate fraction tended to have 24% greater number of cells with undamaged mitochondria and 5% less cells with impaired mitochondria membrane potential than controls ($P < 0.10$, Figure 6D).

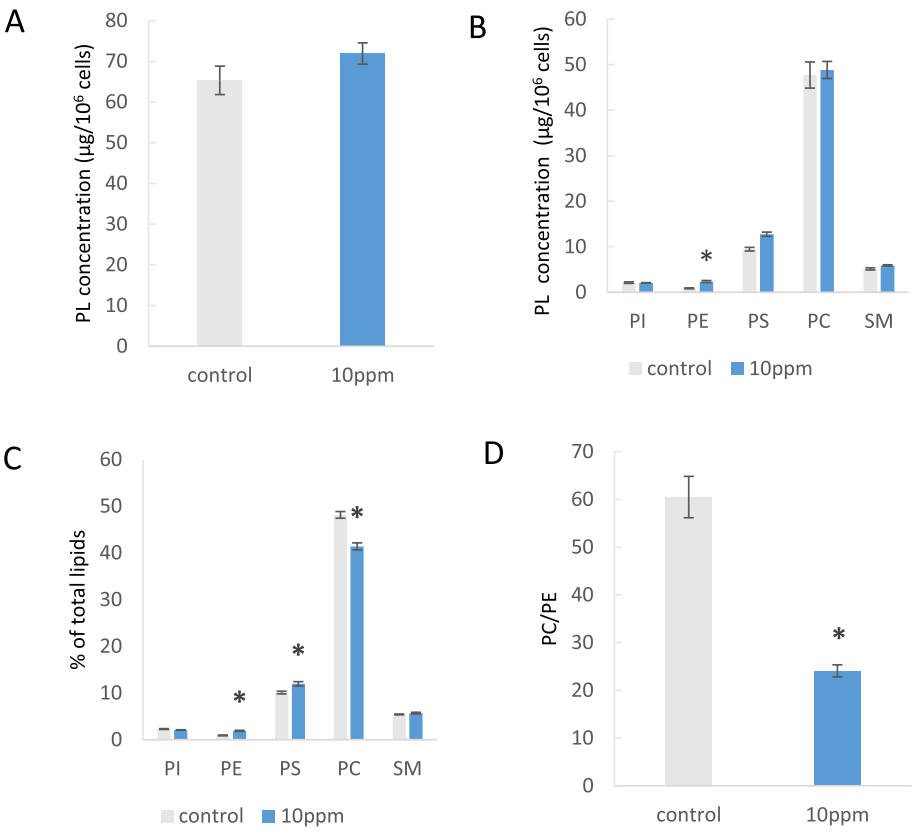

Figure 5. Phospholipids composition of bovine mammary epithelial cells after exposure to 24 h of ethyl acetate fraction obtained from *Pistacia lentiscus* crude ethanol extract in culture medium. After cultivating mammary epithelial cells with 10 ppm *Pistacia lentiscus* ethyl acetate fraction for 24 h, the cells were taken to HPLC to analysis of phospholipids. (A) Phospholipids concentration. (B) Membrane lipid amounts. (C) Phospholipid weight %. The percent of the amount of an individual phospholipid out of the summed phospholipids amounts. (D) Weight ratio between phosphatidylcholine and phosphatidylethanolamine. Data obtained from two independent experiments with 4 replicates for each treatment (n = 4). All data are presented as mean ± SD. * indicate significant differences between treatment to control group ($P < 0.05$). TG: Triglyceride; PL: Phospholipids; PI: Phosphaditylinositol; PE: Phosphaditylethanolamine; PS: Phosphaditylerine; PC: Phosphaditylcholine, SM: Sphingomyelin.

Using the seahorse device, we measured ATP production, cellular respiration, and proton leakage from the mitochondria. Exposure to 10 ppm ethyl-acetate fraction tended to decrease ATP production and basal respiration ($P = 0.07$, Figure 6E).

2.4. MEC exposed to gallic acid and myricetin

Gallic acid and myricetin are both phenolic compounds present in ethyl acetate fraction of lentisk leaves. To test our hypothesis regarding the advantage of whole extract compared with the utilization of isolated polyphenols, the production traits of MEC and their oxygen consumption rate were measured after exposure to these specific phenols in their approximate concentration in 10 ppm ethyl acetate fraction.

Exposure to 2 ppm gallic acid and 1 ppm myricetin did not affect the extracellular content of triglycerides and lactose compared with control ($P = 0.53$ and $P = 0.94$, respectively, Figure 7A,B). However, MEC treated with 10 ppm ethyl acetate fraction which contains 2 ppm gallic acid and 1 ppm myricetin, showed increased lactose secretion ($P < 0.05$, Figure 7B). Milk proteins casein and whey secretion were 126% and 124% higher in after exposure to 1 ppm myricetin and 10 ppm ethyl acetate fraction ($P < 0.05$, $P = 0.05$, respectively, Figure 7C,D), while exposure to 2ppm gallic acid did not change protein secretion compared to control ($P = 0.98$, Figure 7C,D). Exposure to gallic acid and myricetin did not affect intracellular triglyceride content and milk fat globules size, whereas exposure to 10 ppm ethyl acetate fraction increased triglycerides content and milk fat globule size ($P < 0.05$, $P < 0.05$, respectively, Figure 8B,C).

In terms of oxygen consumption, exposure of MEC to 10 ppm of ethyl acetate fraction decreased basal respiration and ATP production by 45% compared with controls ($P < 0.05$, $P < 0.05$, respectively, Figure 9). In contrast, exposure of MEC to gallic acid and myricetin did not affect basal respiration (Figure 9) or ATP production (Figure 9).

3. Discussion

We evaluated the effect of different *P. lentiscus* fractions on production traits and energy-production efficiency in MEC. Using a reductionist approach, bioactive components were extracted based on their polarity, and their effects on MEC production traits were evaluated. In addition, a direct comparison between the ethyl-acetate fraction and its specific phenolic compounds (i.e., gallic acid and myricetin) was conducted, aimed at identifying the bioactivities achieved by the major polyphenolic components of *P. lentiscus*, and at evaluating whether additive or synergistic effects on MEC production traits are elicited by these polyphenols.

In dairy cows, elevated milk production during early lactation is accompanied by an increase in oxidative stress, as suggested by increasing serum peroxidation products such as malondialdehyde, and ROS-neutralizing molecules such as selenium and GSH-Px (Gong and Xiao, 2016). We hypothesized that ROS production and accumulation are elevated in MEC due to the energy required to support biosynthesis and secretion of milk constituents. This may impair cellular activity, by either limiting energy metabolism or challenging cellular resources to mitigate the detrimental effect of ROS. Hence, we sought to treat MEC with active ingredients from the foliage of *P. lentiscus*, with the aim of altering the balance between production and ROS neutralization and allowing cells to generate more energy and redirect it to production. Active compounds from each fraction were characterized and quantified, and their antioxidant capacity determined.

In the present study we used different solvents to produce different extracts. The result was a different composition of phenolic compounds in each extract. We found higher phenolic extract efficiency when using more polar solvents in the following order: hexane < chloroform < ethyl acetate (Table 1). The foliage of *P. lentiscus* was characterized by the presence of phenolic acids such as gallic acid, and in particular, flavonoids such as myricetin derivatives (Azaizeh et al., 2015; Romani et al., 2002; Umadevi et al., 1988). Accordingly, the fractions produced in the

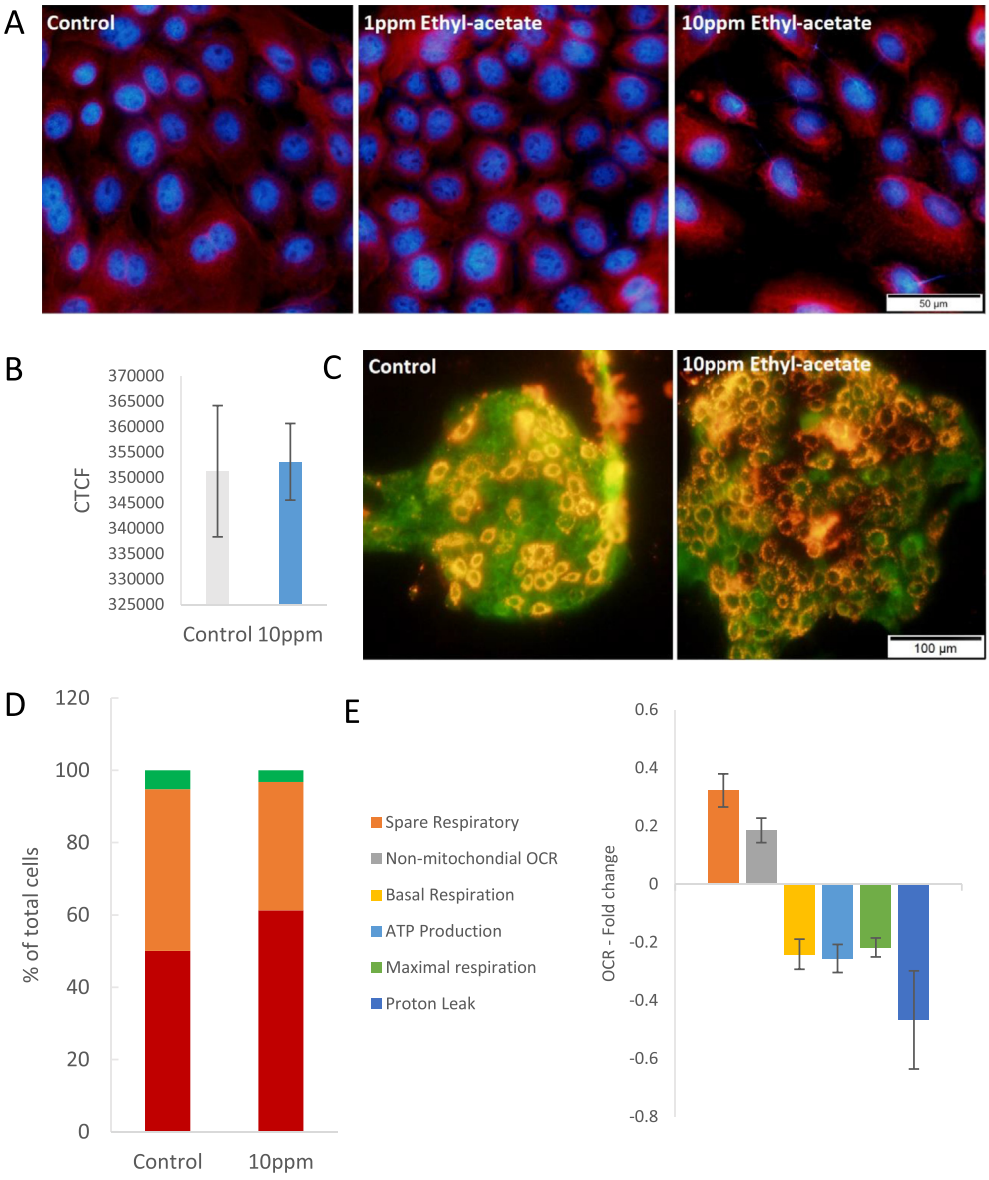

Figure 6. Mitochondria quantity, mitochondria membrane potential and cellular oxygen consumption rate of bovine mammary epithelial cells after exposure to 24 h of ethyl acetate fraction obtained from *Pistacia lentiscus* crude ethanol extract in culture medium. After cultivating mammary epithelial cells with 10 ppm *Pistacia lentiscus* ethyl acetate fraction for 24 h, mitochondria were stained with Mito-tracker, Oxygen consumption rate were measured in Seahorse instrument, and after exposure to H_2O_2 for 2 h, the mitochondria were stained in JC-1. (A) Representative images showing the mitochondria phenotype after mitotracker fluorescent staining (Dapi – Blue, Mitotracker – Red). (B) fluorescence level around mammary epithelial cells nucleus. Data obtained from one experiment with 3 replicates for each treatment (n = 3) for each replicate 10 images were analyzed. (C) Representative images showing the mitochondria potential after JC-1 fluorescent staining (High potential – Red, medium potential – Orange, low potential – green). (D) Mammary epithelial cells distribution to different groups. Data obtained from two independent experiments with 1 replicate for each treatment (n = 1) for each replicate. Ten images were analyzed for each replicate. (E) Oxygen consumption rate as measured by seahorse. Data obtained from two independent experiments with 5 replicates for each treatment (n = 5). Differences in OCR between treatment are expressed in fold of change compared with control. All data are presented as mean ± SD. * indicate significant differences between treatment to control group ($P < 0.05$).

present study from *P. lentiscus* contained gallic acid, except for the hexane fraction. Another compound was ethyl gallate, which was found in all *P. lentiscus* fractions, with the highest concentration in the chloroform fraction. *P. lentiscus* foliage extracts also contained rutin (Azaizeh et al., 2015; Remila et al., 2015), which was found as a major component of the ethyl-acetate fraction but not of the hexane or chloroform fractions.

Neutralization of free radicals by the *P. lentiscus* fractions was determined in vitro by DPPH analysis (Mensor et al., 2001). Most of the fractions of *P. lentiscus* were expected to exert a high level of neutralization (Cherbal et al., 2012; Djeridane et al., 2006). The ethyl-acetate fraction was the most efficient one (Table 1), in accordance with previous studies (Atmani et al., 2009).

Exposing MEC to the various lentisk extracts, we distinguished between the effects on intracellular vs. secreted milk constituents—protein, lactose, and fat. Exposure of MEC to the hexane fraction, containing only ethyl gallate at a low concentration, did not affect MEC production indices at any concentration compared to the control (Figure 1). In contrast, exposure to 10 ppm chloroform fraction, containing a high concentration of ethyl gallate and low concentrations of gallic acid and 3,4-hydroxybenzoic acid, significantly increased triglyceride and casein secretion and showed a tendency to increase secreted whey protein and lactose compared to controls (Figure 1). Exposure to 10 ppm ethyl-acetate fraction significantly increased the secretion of lactose and both casein and whey proteins, with no change in triglyceride secretion (Figure 1). These results aligned with the antioxidant capacities of the various fractions: the hexane fraction showed inferior antioxidant capacity and accordingly, was less able to increase the biosynthetic properties of the MEC. The fact that only the ethyl-acetate fraction increased lactose content in the cells suggests that the improved antioxidant capacity released glucose from the generation of reduction elements needed to neutralize ROS and channeled it to lactose synthesis. Nevertheless, the fact that triglyceride secretion was positively affected by the chloroform but not the ethyl-acetate fraction suggests that the cells' capacity to secrete triglycerides is independent of the antioxidant capacity of the extract treatments. These results therefore suggest an additional mode of action for polyphenols that does not depend on their antioxidant activity. Moreover, the fact that triglyceride secretion was affected only by the chloroform fraction suggests that ethyl gallate, which was present at high concentrations in this fraction, plays a role in these processes. On the other hand, measurement of intracellular triglycerides showed a significant increase compared to the control group after exposure to the ethyl-acetate fraction, whereas their concentration did not change after

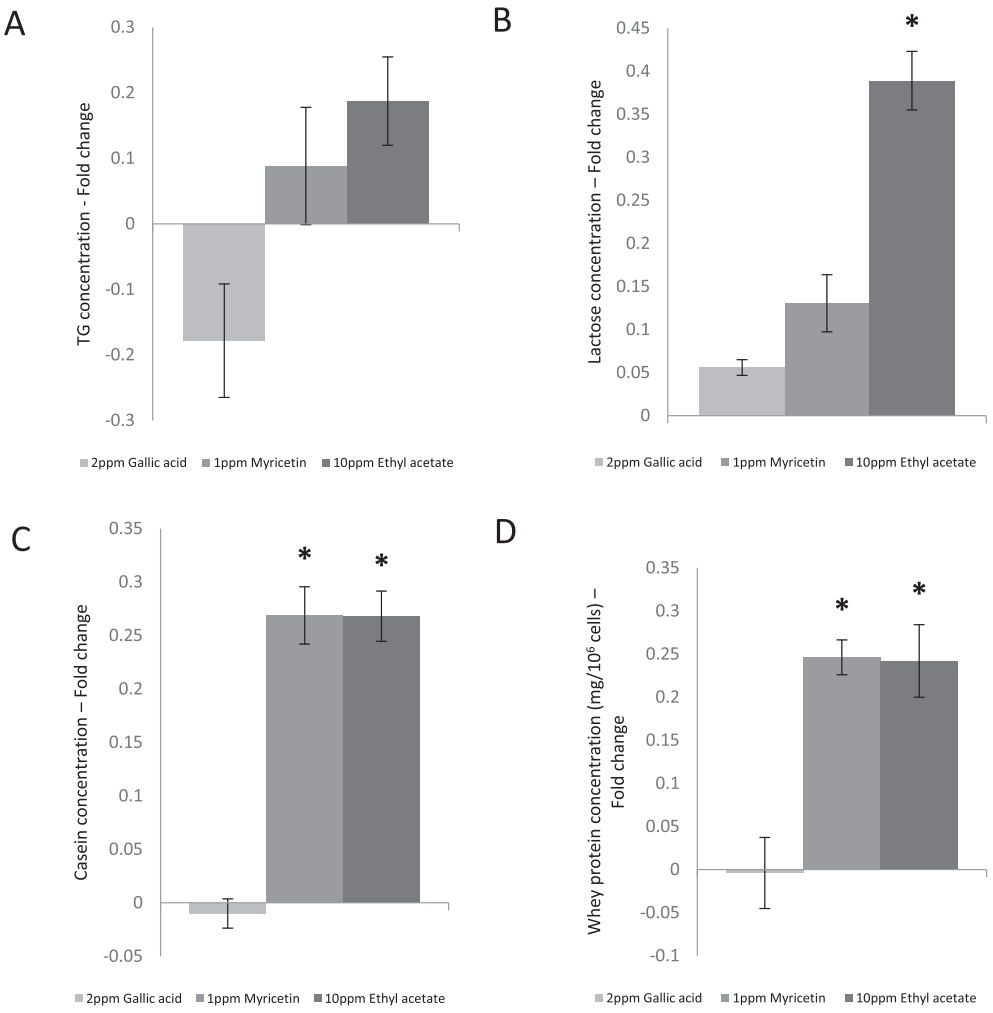

Figure 7. Extracellular manufacturing properties of bovine mammary epithelial cells after exposure to 24 h Gallic acid, myricetin or ethyl acetate fraction obtained from *Pistacia lentiscus* crude ethanol extract in culture medium. After cultivating mammary epithelial cells with 2ppm gallic acid, 1 ppm myricetin and 10 ppm *Pistacia lentiscus* ethyl acetate fraction for 24 h, the medium was collected and taken to HPLC to analysis of Triglyceride, Protein and lactose. (A) Triglyceride concentration. (B) Lactose concentration. (C) Casein protein concentration. (D) Whey protein concentration. Differences in triglycerides, lactose casein and whey protein content between treatments are expressed in fold of change compared with control. Data obtained from two independent experiments with 5 replicates for each treatment ($n = 5$). All data are presented as mean ± SD. * indicate significant differences between treatment to control group ($P < 0.05$).

exposure to the chloroform fraction (Figure 2C). These results imply that the effect of the different fractions is related to triglyceride secretion rather than its synthesis.

Lipid-droplet size was elevated in MEC treated with the ethyl-acetate fraction, with a concomitant increase in PE amount and relative concentration, and a decrease in the concentration of PC (Figure 3C). These changes in the lipid profile caused a significant decrease in the PC:PE ratio in cells exposed to 10 ppm ethyl-acetate fraction (Figure 3D). PE and PC contents, and specifically their mass ratio, have been found to affect membrane stability (Thiam et al., 2013), making intracellular lipid droplets more prone to fusion (Argov-Argaman, 2019). This mechanism has been validated in MEC primary culture and has been found to affect the diameter of intracellular lipid droplets and secreted milk fat globules (Cohen et al., 2015, 2017). Another factor that affects lipid droplet size is cellular triglyceride content, which was elevated upon exposure to 10 ppm ethyl-acetate fraction with no change in the total phospholipid content (Figure 2B). This phenotype requires the formation of larger lipid droplets to envelop the greater triglyceride content with the same membrane content. These results are in accordance with a previous study in which limited PC content in hepatocytes increased the size of the lipid droplets (Guo et al., 2008). They suggest that the modulation of lipid droplet size that occurs in MEC treated with the ethyl-acetate fraction of *P. lentiscus* leaves can be attributed to a combination of increasing lipogenic activity in the cells and enhanced fusion between lipid droplets.

A change in PE content is associated with a change in mitochondrial activity and number (Cohen et al., 2017). The effect of certain polyphenols on mitochondrial quantity has been shown in hepatocytes. For example, Rafiei et al. (2017) found an increase in mitochondrial biogenesis in hepatocytes 2 h after exposure to resveratrol and quercetin, whereas catechin did not affect mitochondrial quantity. Resveratrol and quercetin have also been found to increase mitochondrial biogenesis in endothelial, muscle and brain cells (Csiszar et al., 2009; Davis et al., 2009; Davinelli et al., 2013). In the present study, 24-h exposure to the ethyl-acetate fraction, containing seven different polyphenols, did not affect the number of mitochondria detected (Figure 3B). This might be due to the absence of the specific polyphenols, for example quercetin, that induce mitochondrial biogenesis.

Although no change in the number of mitochondria was observed, a tendency toward changes in MEC oxygen consumption and ATP production was recorded upon exposure to the ethyl-acetate fraction (Figure 3E). To support the greater production of milk constituents, higher metabolic rates were expected. However, we found that although MEC treated with the ethyl-acetate fraction produced more milk components, they used less oxygen and produced less ATP. These results confirmed that plant extracts can boost mitochondrial efficiency. This greater efficiency may be attributed to the cells' capacity to maintain stable oxidative status, even though greater amounts of ROS are produced due to elevated metabolic activity. To test this hypothesis, cells were exposed to pro-oxidative medium using hydrogen peroxide. After exposure, the cells were stained with JC-1, an indicator of mitochondrial membrane potential. We found that a greater percentage of the cells maintained active mitochondria after treatment with the ethyl-acetate fraction compared to the control (Figure 3D), hence supporting greater production of milk components.

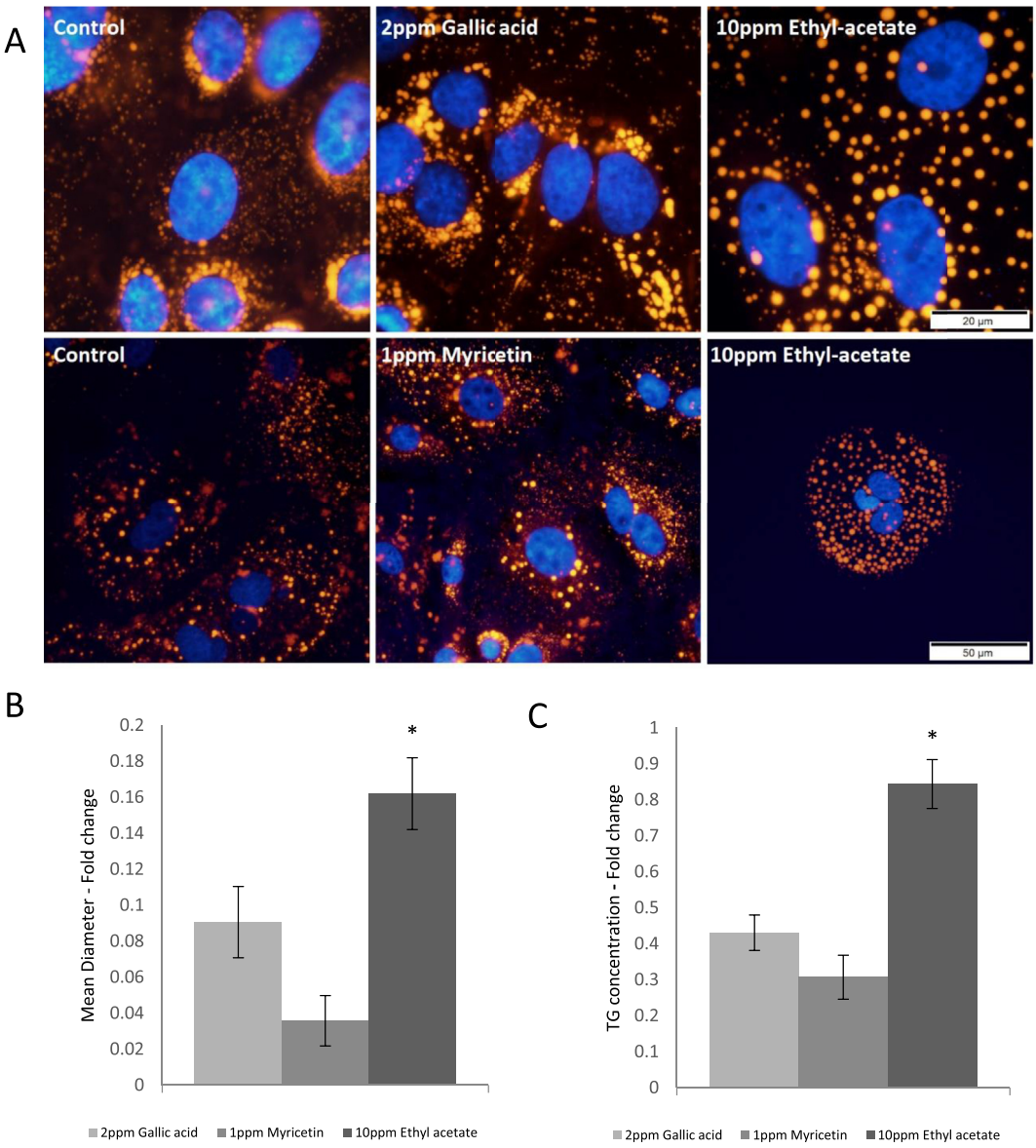

Figure 8. Intracellular manufacturing properties of bovine mammary epithelial cells after exposure to 24 h Gallic acid, myricetin or ethyl acetate fraction obtained from *Pistacia lentiscus* crude ethanol extract in culture medium. After cultivating mammary epithelial cells with 2ppm gallic acid, 1 ppm myricetin and 10 ppm *Pistacia lentiscus* ethyl acetate fraction for 24 h, intracellular lipid droplets stained in Nile red, the cells were taken to HPLC to analysis of triglyceride. (A) Representative images showing the intracellular phenotype after lipid droplets fluorescent staining (Dapi – Blue, Nile red – Yellow). (B) After staining lipid droplets, the droplets were counted, and the mean diameter was measured. Data obtained from one experiment with 3 replicates for each treatment (n = 3); Ten images were analyzed for each replicate. (C) Triglyceride concentration. Differences in lipid droplets diameter and triglycerides content between treatment are expressed in fold of change compared with control. Data obtained from two independent experiments with 4 replicates for each treatment (n = 4). All data are presented as mean ± SD. * indicate significant differences between treatment to control group ($P < 0.05$).

The results of the present study suggest that the effects of polyphenols on MEC production traits are not limited to their antioxidant capacity. Moreover, they suggest that the different polyphenol constituents contribute to these traits in specific and different ways. To examine this hypothesis, we exposed MEC to 10 ppm ethyl-acetate fraction, or to the specific polyphenols in that fraction—gallic acid or myricetin—at concentrations similar to their concentrations in the ethyl-acetate fraction.

Exposure to myricetin alone increased the secretion of both casein and whey proteins to the same extent as exposure to 10 ppm ethyl-acetate fraction. In contrast, exposure to 2 ppm gallic acid did not boost protein secretion (Figure 5), indicating that the effect of the ethyl-acetate fraction on protein secretion can most probably be attributed to myricetin. Studies have previously shown antioxidant activity for myricetin (Wang et al., 2010) and for gallic acid (Deng et al., 2011; Badhani et al., 2015). However, only myricetin contributed to the production response, which reinforces the hypothesis that the polyphenols' effects go beyond the simple action of neutralizing oxidizing compounds. In addition, unlike the effect of the ethyl-acetate fraction, exposing MEC to either gallic acid or myricetin did not affect the secretion of lactose or triglycerides, although a tendency toward increased triglyceride content in the cells and milk fat globules size was found (Figure 6). Taken together, the effects of isolated gallic acid and myricetin were only marginal compared to the effects elicited by exposure to the ethyl-acetate fraction. These results suggest that polyphenols have an additive effect on productivity traits or alternatively, an entourage effect, i.e., one compound has no influence on its own but in combination with another compound, will influence productivity. The additive effect of polyphenols was reflected in the cells' oxygen consumption (Figure 7). Although not statistically significant, exposure to myricetin or gallic acid tended to lower ATP production and cellular respiration compared to the control, whereas

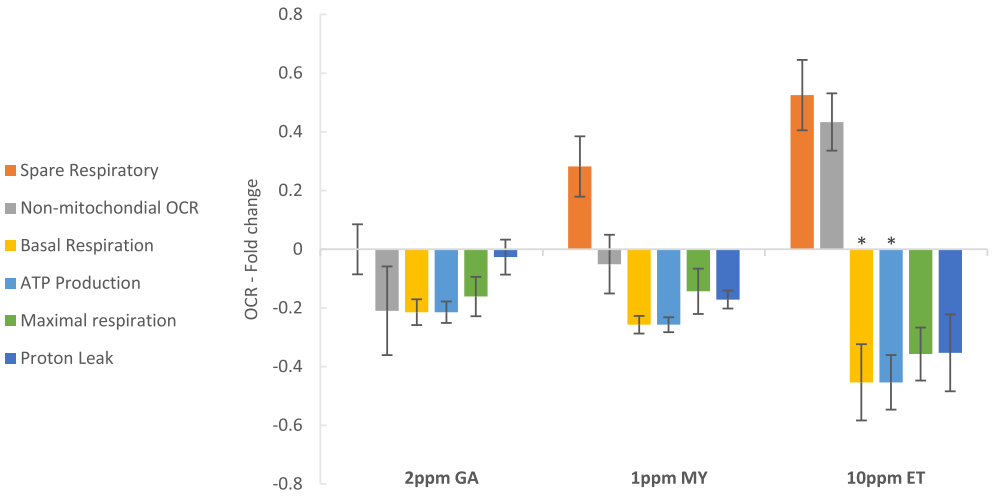

Figure 9. Cellular oxygen consumption rate of bovine mammary epithelial cells after exposure to 24 h gallic acid, myricetin or ethyl acetate fraction obtained from *Pistacia lentiscus* crude ethanol extract in culture medium. After cultivating mammary epithelial cells with 2ppm gallic acid, 1 ppm myricetin and 10 ppm *Pistacia lentiscus* ethyl acetate fraction for 24 h, Oxygen consumption rate was measured in Seahorse instrument. Differences in OCR between treatments are expressed in fold of change compared with control. Data obtained from two independent experiments with 5 replicates for each treatment (n = 5). All data are presented as mean ± SD. * indicate significant differences between treatment to control group ($P < 0.05$).

Table 4. Production traits summary.

Treatment	Polyphenol content	MFG Diameter	Triglycerides		Lactose	Casein	Whey protein
			In	Out			
0.1 ppm Hexane	100% Ethyl gallate	-	-	-	-	-	-
10 ppm Chloroform	95.25% Ethyl gallate	-	-	↑	-	↑	-
	2.75% Benzoic acid						
	2% Gallic acid						
10 ppm Ethyl acetate	46.67% Rutin	↑	↑	-	↑	↑	↑
	17.59% Gallic acid						
	10.8% Ethyl gallate						
	10.38% Myricetin						
	7.75% Syringic acid						
	5.8% Catechin						
	0.96% Benzoic acid						
2 ppm Gallic acid	100% Gallic acid	-	-	-	-	-	-
1 ppm Myricetin	100% Myricetin	-	-	-	-	↑	↑

exposure to the ethyl-acetate fraction lowered these indices significantly. These results thus imply an additive effect of the polyphenols that are administered as a whole extract compared to their administration as isolated compounds. The positive effects can also be attributed to rutin, which is one of the major components of the ethyl-acetate fraction.

4. Conclusion

Increased synthesis and secretion in MEC were found to be associated with the antioxidant capacity of the different lentisk fractions (Table 4). Nevertheless, this may not be the only mechanism contributing to MEC productivity. An examination of mitochondrial activity revealed an increase in the respiration potential of the cells, with no change in the number of mitochondria. While the mechanism is not entirely clear, these findings suggest improved efficiency in mitochondrial function, concomitant with enhanced milk-production properties. Finally, it should be pointed out that specific polyphenol compounds had different effects on milk-production properties, implying differential potential for application.

5. Materials and methods

5.1. Material

DMEM/F12, fetal bovine serum (FBS), penicillin, streptomycin, amphotericin B, L-glutamine solution, trypsin–EDTA solution C and L-methionine were purchased from Biological Industries (Beit Haemek, Israel). Bovine insulin, hydrocortisone, ovine prolactin, bovine serum albumin (BSA) solution, hyaluronidase, DNase I, heparin, PE (1,2-dioleoyl-sn-glycero-3 phosphoethanolamine, 10 mg PL per mL $CHCl_3$, purity 99%), PI (L-α phosphatidylinositol ammonium salt, from bovine liver, purity 98%), PS (1,2-dioleoyl-sn-glycerol-3-phospho-L-serine sodium salt, purity 95%), PC (1,2-dioleoyl-sn-glycero-3-phosphocholine, purity ≥99%) and SM (sphingomyelin; from bovine brain, purity 97%). Gallic acid and myricetin were purchased from Sigma Aldrich Israel Ltd. (Rehovot, Israel). Collagenase type II was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Sodium azide, sodium fluoride and DZA were purchased from Sigma Aldrich. All seahorse device products were purchased from Agilent technologies© (CA, USA).

5.2. Extracts

Lentisk foliage was collected during the summer and spring from Ramat Hanadiv Nature Park (south of Carmel Heights, 32°33′N, 34°56′E). Leaves were dried at 50 °C for 24 h, then ground with a laboratory grinder (KM-700, MRC, Israel) to approximate particle size of 2 mm where in each batch 100 g of grinded leaves were extracted with a one liter of 70% ethanol solution for 3 h shaking. Extracts were filtered and evaporated under vacuum (Rotorvapor Hie-VAP; Hiedolph, Germany) at 45 °C to remove the ethanol and water to dryness (Azaizeh et al., 2013). To achieve further fractionation, 20 g of ethanolic extract was exposed to organic solvents with increasing polarity, where the first

solvent was hexane, then, chloroform, and the last ethyl acetate, respectively according to a previous study (Tiwari et al., 2011). Each separation was made with 3 funnels containing 100 ml solvent. The 30 ml of aquatic layer of the lentisk ethanolic extract from the last dry were funneled 3 times, the residual material in the funnel was filtered and dried in a spinning drier at 45 °C, then moved to a spinning evaporator. The extracted fractions were dried at 50 °C under a vacuum oven.

5.3. Phenolic content in the P. lentiscus extracts

Total phenol (TP) content of the *P. lentiscus* fractions was determined according to the Folin-Ciocalteu method (Zhishen et al., 1999). Samples were prepared by dissolving 40 mg from *P. lentiscus* fractions in 100 ml methanol/DDW (50:50). One ml from the fraction or standard samples was mixed with 500 μl Folin and Ciocalteu's phenol reagent. After 3 min, 3 ml of Na_2CO_3 was added and when the mixture developed blue color, 5.5 ml of DDW were added. Then the mixture was placed in a centrifuge at 3500 g for 5 min and absorbance of the supernatant was measured against a blank sample at 740 nm using an Evolution 260 Bio UV–Visible Spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). The concentration of polyphenols was determined by a colorimetric method, based on external calibration curve using caffeic acid (5, 10, 15, 20 and 40 mg/L) in methanol/DDW (50:50). Total flavonoid content was determined according to Zhang et al. (2011). TP were expressed in terms of gallic acid equivalent (mg phenols/g of dry extract), which is considered a reference to a common phenolic compound. Flavonoids in the samples were expressed as quercetin equivalent (mg/g of dry extract), and tannins were expressed as tannic acid equivalent (mg/g dry extract).

5.4. Identification and quantification of phenolic compounds in P. Lentiscus fractions were determined by High Performance Liquid Chromatography (HPLC)

Lentisk fractions were analyzed using HPLC-PAD (HPLC – Thermo scientific Finnigan surveyor) equipped with a detector PDA plus (220–340 nm). Separation was made using Gemini 5 μm C6-Phenyl 110A° (250*4.6mm) column at a temperature of 30 °C, and flow was 1 mL/min for 35 min. An analysis was made using two solvents: 0.1 acetic acid in water (A) and 0.1 acetic acid in methanol (B). Specifically: 0–3 min 25% B; 3–15 min 25–50% B; 15–20 min 50% B; 20–29 min 50-25% B; 29–35 min 25% B. The major phenolic compounds were identified from the different lentisk fractions based on comparison to the column exit time and the UV spectra of pure standards (Tafesh et al., 2011).

5.5. Determination of antioxidant activity by radical neutralization method, using a stable DPPH radical

Antioxidant activity of various lentisk leaf foliage extracts was measured in terms of hydrogen contribution or radical repulsion capacity, using a stable DPPH radical (Brand-Williams et al., 1995). The stock was prepared as follows: 25 mg DPPH was added to 1 L of methanol to obtain a deep purple DPPH stock solution. 3.9 ml solution from the stock added to a 0.1 ml series of different concentrations of the *P. lentiscus* fractions (1–200 ml/liter). The mixtures were incubated at 37 °C for 30 min, after which the absorbance was measured at 517 nm wavelength spectrophotometer. Ascorbic acid was used as a positive control. All series were performed in triplicates. This experiment was repeated twice. The percent delay of sampling by radical-DPPH was calculated according to the formula: Scavenging activity (%) = $[(A_{control} - A_{sample})/A_{control}] * 100$ when $A_{control}$ is the absorption at t = 0 and A_{sample} is the antioxidant absorption at t = 30. The repulsion ability of the various fractions is expressed in the value of EC50, which is the effective concentration at which 50% of DPPH radicals were deposited. The EC50 value was obtained from the graph of impaction activity (%) versus the concentration of samples, and it was calculated by linear regression. A low EC50 value indicates a strong ability of the extract to act as a repellent to DPPH.

5.6. Mammary epithelial cells response to P. lentiscus extracts experimental design

For all the experiments, we used primary culture of bovine mammary epithelial cells isolated from 3 lactating Holstein cows according to a protocol established in our lab (Cohen et al., 2015). Study protocols were in compliance with the regulations of the Israeli Ministry of Health, under the supervision of the Department for control of Animal products, State of Israel Ministry of Agriculture rural development veterinary services and animal health. Certificate Nu: # 80. After isolation, 60,000 cells were plated in a 35-mm plastic dish on glass cover slips for all fluorescence staining experiments. For production analysis, 150,000 cells were plated in a 60-mm plastic dish for lipids, protein, and lactose extraction. Oxygen consumption assay was performed by using Seahorse system (Agilent, USA); cells were plated in 24-well plate (20,000 cells/well) and incubated with DMEM/F12, with 0.15% (v/v) bovine serum albumin and insulin (1 μg/ml), hydrocortisone (0.5 μg/ml) and prolactin (1 μg/ml) for 48 h to induce lactogenic response. Then cells were treated with three different *P. lentiscus* extracts: ethyl acetate, hexane and chloroform, according to the protocol that was used before (Azaizeh et al., 2013). The extracts were diluted with DMEM/F12 medium supplemented with Dimethyl Sulfoxide (DMSO) in a final concentration of 0.1% for better dissolution of the extractions. Oleic acid was added to the treatment medium at a final concentration of 0.1 M to increase fat (triglyceride) synthesis to better enable the visualization of intracellular lipid droplets. The treatment was given in the presence of insulin (1 μg/ml), hydrocortisone (0.5 μg/ml) and prolactin (1 μg/ml). After 24h of incubation, medium was collected for triglyceride and phospholipid quantification, and cells were harvested for cell counting, lipid, lactose or protein extraction. Otherwise, cells were fixed for fluorescence staining or plates taken to seahorse for oxygen consumption rate measurement.

5.7. Intracellular lipid droplets fluorescence staining

After treatment, cells grown on glass were rinsed three times with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After fixation, the cover slips were rinsed three times with PBS and stained with Nile red (200 nM, sigma) for 15 min. Then cover slips were rinsed three times with PBS and stained with DAPI (Sigma, St. Louis, MO) for 5 min. Cover slips were then rinsed four times with PBS and mounted with fluorescence mounting medium (Dako, North America Inc., Carpinteria, CA). Slides were visualized with an Olympus BX40 fluorescence microscope equipped with an Olympus DP73 digital camera using CellSens Entry software (version 1.7, Olympus). Lipid droplet diameter was measured using ImageJ software (version 1.48, NIH, Bethesda, MD). The droplets were measured only after they stood in parameter of 0.1–1 circularity, and the aspect ratio was smaller than 1.5 to avoid artifacts. Differences in lipid droplets diameter between treatment are expressed in fold of change compared with control. Positive values indicate increased diameter whereas negative values indicate decreased diameter.

5.8. Determination of mitochondria amount

After treatment, cells were incubated in DMEM/F12 with 500 nM Mitotracker deep red FM immunostaining (Cell Signaling Technology) for 30 min at 37 °C. The cells were then fixed in ice-cold 100% methanol for 15 min at -20 °C and rinsed three times with PBS. Cells were mounted with a fluorescence mounting medium (Dako), and slides were visualized with an Olympus BX40 fluorescence microscope equipped with an Olympus DP73 digital camera using CellSens Entry software version 1.7. Mitochondrial fluorescence in each cell was quantified by ImageJ software version 1.48 using the following formula: Corrected total cell

fluorescence = Integrated density of selected cell (Area of selected cell x Mean fluorescence of background readings).

5.9. Determination of mitochondrion membrane potential

After treatment on glass cover slips, cells were incubated with H_2O_2 to establish *in vitro* oxidative stress. Freshly before being used, 30% H_2O_2 was diluted to 0.1M stock using a sterilized PBS. Further, 1M H_2O_2 was diluted with cell culture medium to a final concentration of 0.5mM for 2h. Then, in order to measure mammary epithelial mitochondrial membrane potential ($\Delta\Psi m$), cells were incubated for 10 min at 37 °C with 153μM of 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide fluorescent probe (JC-1; ENZO life sciences international, Plymouth Meeting, PA, USA). Cells were then rinsed three times with PBS and visualized with an Olympus BX40 fluorescence microscope equipped with an Olympus DP73 digital camera using CellSens Entry software version 1.7. Mitochondrial membrane potential fluorescence in each cell was qualified by ImageJ software version 1.48 by three colors: red – high potential, orange – moderate potential and green – low potential.

5.10. Milk lipid extraction and analysis

After treatment, total lipids were extracted from harvested cells and from collected medium. The medium or the cells were diluted by 20 times Folch reagent (Chloroform-Methanol 2:1). After overnight incubation with cold extraction at 4 °C, the upper phase was removed, and the lower phase was filtered through glass wool. Samples were then evaporated under a nitrogen stream at 65 °C, diluted in 100 μl chloroform:methanol (97:3, v/v) and stored at -20 °C until injection for HPLC analysis. Separation of polar and neutral lipids was performed on a silica column (Zorbax RX-SIL, 4.6 × 250 mm, Agilent Technologies) using HPLC (HP 1200, Agilent Technologies, Santa Clara, CA) with an evaporative light-scattering detector (1200 series ELSD, Agilent Technologies). The column was heated to 40 °C, flow was 1 mL/min, and injection volume was 20 μl. The ELSD was heated to 65 °C, nitrogen pressure was 3.8 bars, filter level was set on 5, and gain (sensitivity) was set to 4 for the first 14 min, then changed to 12 until 21 min, and then changed to 7 until the end of the run, to enable detection of differently abundant lipid components. The separation protocol consisted of a gradient of dichloromethane, methanol: ammonium mix (99:1, v/v), and double-distilled water. The separation process was managed by ChemStation software (Agilent Technologies), which permitted the acquisition of data from the ELSD detector. The separated lipids were identified using external standards (Sigma Aldrich). Quantification was performed against external standard curves and expressed as μg/per 10^6 live cells or as weight% out of the sum of phospholipids (μg) in the sample. Live cell number was determined with a hemocytometer after 5 min of Trypan blue staining. Differences in triglycerides between treatment are expressed in fold of change compared with control. Positive values indicate increased triglycerides content whereas negative values indicate decreased triglycerides content.

5.11. Milk protein extraction and analysis

After treatment, 0.5 ml cultured medium was collected to HPLC equipped with UV detector. Protein content was determined in 220 nm wavelength by C-18 reverse phase column. Elution gradient was 1 ml\min at 30 °C for 30 min; injected volume was 20 μl. Mobile phase composition mixed acetonitrile: water: trifluoroacetic acid (100: 900: 1, v/v/v) as solvent A and (900: 100: 1) as solvent B. Specifically, 1–4 min, 100% of A solvent; 4–20 min, 100-25% of A solvent; 20–24 min, 25% of A solvent; 24–26 min, 25–100% of A solvent; 26–30 min, 100% of A solvent. Identification and quantification were determined by constructing a calibration curve of external standard of known protein concentrations which dissolved in a 50 mM phosphate buffer (PH = 6.7). Identification for α casein, β casein and α lactalbumin were qualified as caseins (α and β casein) and whey (α lactalbumin) protein. Calibration curve strength for caseins and whey were $R^2 = 0.94$ and $R^2 = 0.96$, respectively. Differences in protein between treatment are expressed in fold of change compared with control. Positive values indicate increased protein content whereas negative values indicate decreased protein content.

5.12. Milk lactose extraction and analysis

After treatment, 0.5 ml medium was collected to be analyzed using HPLC equipped with a refractive index detector at 68 °C. Sulfuric acid (0.005N) was used for elution in 0.6 ml/min for 14 min in Rezex-ROA-acids H^+ column. Identification and quantification were determined by establishing a calibration curve of external standard of a known lactose concentration dissolved in water. Calibration curve strength for lactose was $R^2 = 0.99$. Differences in lactose between treatment are expressed in fold of change compared with control. Positive values indicate increased lactose content whereas negative values indicate decreased lactose content.

5.13. Oxygen consumption rate and analysis

After treatment, oxygen consumption rate (OCR) of live cells was measured in real time using a Seahorse Bioscience XF24 extracellular flux analyzer (North Billerica, MA). Then, cell growth medium was substituted to XF assay media (pH 7.4, Seahorse Biosciences) supplemented with 2 mM glutamine and 1 mM sodium pyruvate. Prior to the cellular respiration measurement, cells were incubated for 1 h at 37 °C without CO_2. The inhibitors of mitochondrial respiration, including oligomycin at 2 μM, carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) at 0.5 μM and rotenone at 0.5 μM were auto-injected into the experimental wells after basal measurements, and another three measurement cycles were performed. Each experimental point is an average of a minimum of four replicate wells. Immediately after finishing the flux measurements, cells were counted by a hemocytometer, and the values were used for normalization of the OCR values. Differences in OCR between treatment are expressed in fold of change compared with control. Positive values indicate increased OCR whereas negative values indicate decreased OCR.

5.14. Statistical analysis

All statistical analysis was performed using JMP software version 14.0.0 (SAS Institute, Cary, NC, USA). All reported data are means ± SD. All dependent variables were checked for homogeneous variance by unequal variances in JMP software, and if the variance was not homogeneous, a Welch–ANOVA test was performed. The effect and comparisons between treatments were tested by ANOVA followed by LS-Mean Tukey-Kramer HSD multiple-comparison test. The distribution of cell phenotypes based on mitochondrial potential membrane categories, and the distribution of lipid droplet size categories was compared by Chi square test. Significance probe was set to 0.05 and tendencies were reported at $0.05 < P \leq 0.1$.

Declarations

Author contribution statement

Y. Shalev: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

O. Hadaya, H. Muklada, T. Glasser: Performed the experiments.

R. Bransi-Nicola: Performed the experiments; Analyzed and interpreted the data.

S.Y. Landau, Z. Roth: Conceived and designed the experiments.

H. Azaizeh: Contributed reagents, materials, analysis tools or data.

T. Deutch-Traubman: Contributed reagents, materials, analysis tools or data.

M. Haj-Zaroubi: Analyzed and interpreted the data.

N. Argov-Argaman: Conceived and designed the experiments; Wrote the paper.

Funding statement

This work was supported by the Chief Scientist of the Israeli ministry of Agriculture (0922-001).

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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