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# *In vitro* fermentation of cardoon seed press cake - A valuable byproduct from biorefinery as a novel supplement for small ruminants

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ABSTRACT

The multipurpose cardoon crop (*Cynara cardunculus* var. *altilis*) is also exploited to produce bio-intermediates for biorefinery; the press cake composed by partially defatted seed residue after oil extraction represents an important byproduct. This research was addressed at elucidating feeding value and traits of cardoon press cake to be exploited as a novel supplement for feeding ruminants. Specific aims were: to (i) determine the nutritional profile of press cake and (ii) evaluate the effect of anti-tannic agents on methane production, ammonia level, ruminal volatile fatty acids profile, ruminal biohydrogenation of linoleic and linolenic acids, crude protein (CP) degradation and fiber (NDF) digestion. Cardoon press cake shows moderate level of CP (18.5%) and fat (7.9%) with high level of NDF, and represents a valuable source of both essential amino acids and n-6 fatty acids. The content of phenolics reached 32.7 mg $g^{-1}$ and could positively affect the transfer of n-6 unsaturated fatty acids (−50% of biohydrogenation) in milk and meat ruminant products without negative effects for CP degradability and NDF digestibility. In addition, it is able to modulate the methane release, in particular 48 h after incubation. A moderate level of the polyphenol oxidase was also detected. *In vitro* results indicate that cardoon press cake is suitable for animal feeding and for improving their performances and products with potential benefits for human consumers, due to its content of unsaturated fatty acids with a proper amount of phenolic compounds.

## 1. Introduction

*Cynara cardunculus* var. *altilis* (cardoon) has achieved increasing interest and economic value in Mediterranean environments for multipurpose uses. Cardoon, belongs to Asteraceae family, is a drought resistant perennial species, having an annual growth cycle with vegetative stasis in summer, and can be grown under rainfed regime and low-quality soils (Ledda et al., 2013; Mauromicale et al., 2014). Biomass fractions from the entire cardoon plant can be exploited for different applications, to produce green forage, lignocellulosic feedstock, solid and liquid biofuels, paper industry pulp, inulin, and bioactive compounds with antimicrobial, pharmaceutical and antioxidant properties (Cajarville et al., 1999; Fernández et al., 2006; Raccuia and Melilli, 2007; Grammelis et al., 2008; Pandino et al., 2011; Gominho et al., 2011; Oliveira et al., 2012; Ramos et al., 2013; Gominho et al., 2018).

Recent experiments found that the yield of achenes (*i.e.*, cardoon fruits also referred to as seeds) ranged from 0.6 to about 3 t $ha^{-1}$ (Vasilakoglou and Dhima, 2014; Francaviglia et al., 2016; Neri et al., 2017; Ottaiano et al., 2017). In particular, the achenes can be exploited not only to produce edible oil and biodiesel, but also to supply important bio-intermediates for biorefinery.

As a result of the first processing phase dealing with the mechanical oil extraction from cardoon achenes, the partially defatted oil seed solid residue, seed press cake (thereafter referred to as press cake) represents an important biorefinery byproduct, which can be used for animal feeding (Curt et al., 2010; Genovese et al., 2016) or as soil amendment to control soil-borne plant pathogens (Pugliese et al., 2017; De Corato et al., 2018).

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**Table 1**
Nutritional profile and protein fractions of cardoon press cake (average ± standard deviation).

| Items | Units of measurements | Average value |
|---|---|---|
| DM | % | 96.91 + 0.15 |
| CP | % DM | 18.52 + 0.08 |
| EE | " | 7.95 + 0.30 |
| Ash | " | 6.01 + 0.06 |
| NDF | " | 53.45 + 0.16 |
| ADF | " | 47.29 + 0.11 |
| ADL | " | 13.47 + 0.45 |
| Starch | " | 1.22 + 0.30 |
| *Protein fraction* | | |
| TP | % CP | 81.76 + 3.09 |
| NDIN | " | 18.49 + 0.71 |
| ADIN | " | 18.36 + 0.39 |
| SP | " | 58.97 + 1.84 |

DM = dry matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin; TP = true protein; NDIN = neutral-detergent insoluble nitrogen; ADIN = acid-detergent insoluble nitrogen; SP = soluble protein ($A + B_1$).

A complex of green chemistry for the production of bio-lubricants, bio-plastic and additives from integrated biorefinery in local areas has been established in Sardinia (Italy) and an innovative agricultural production chain based on the cultivation of cardoon crop has been developed. In meantime, the traditional dairy sheep farming system in the same area requires remarkable amounts of protein supplements from the market, which are produced overseas and then imported each year. Therefore, the availability of cardoon press cake can represent not only an important feed source option but also a valuable example of integration between green chemistry industry and traditional farming (Centi and Perathoner, 2012; Cravero et al., 2012).

Nevertheless, information regarding the chemical composition and feeding value of cardoon press cake for ruminant nutrition is scanty and needs to be updated. Previous works (Cajarville et al., 2000; Genovese et al., 2016) have shown that both cardoon whole seed and press cakes are characterized by moderate level of crude protein (CP), high level of lignin (ADL) and low fat (EE) than linseed press cake. In addition, Falleh et al. (2008) have underlined that cardoon seeds could be a source of phenolic compounds, which could interact with animal feed efficiency in terms of crude protein utilization and ruminal biohydrogenation (Cabiddu et al., 2009; Piluzza et al., 2014). In order to better evaluate the relationship between phenolics activity and protein

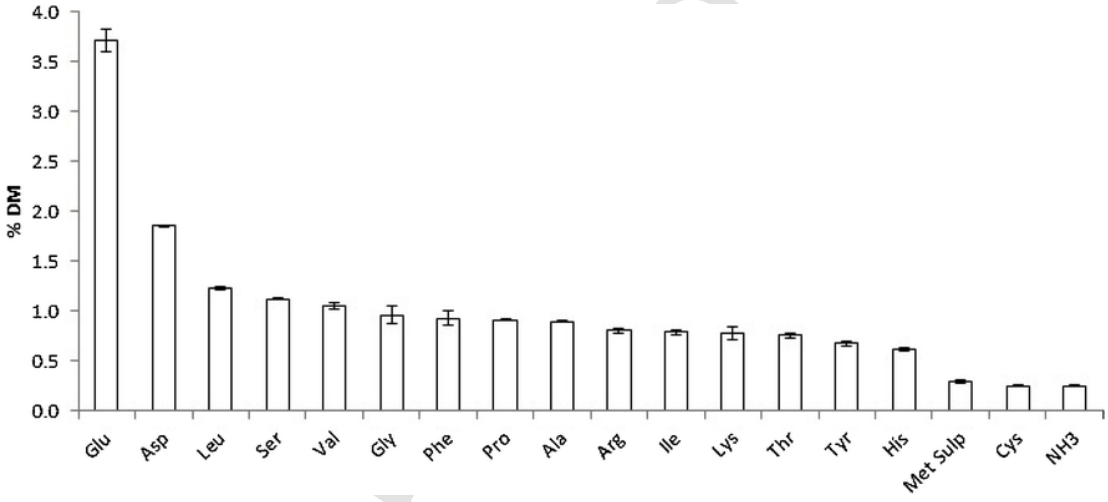


**Fig. 1.** Amino acid profile of cardoon press cake (Vertical bars represent standard deviations).

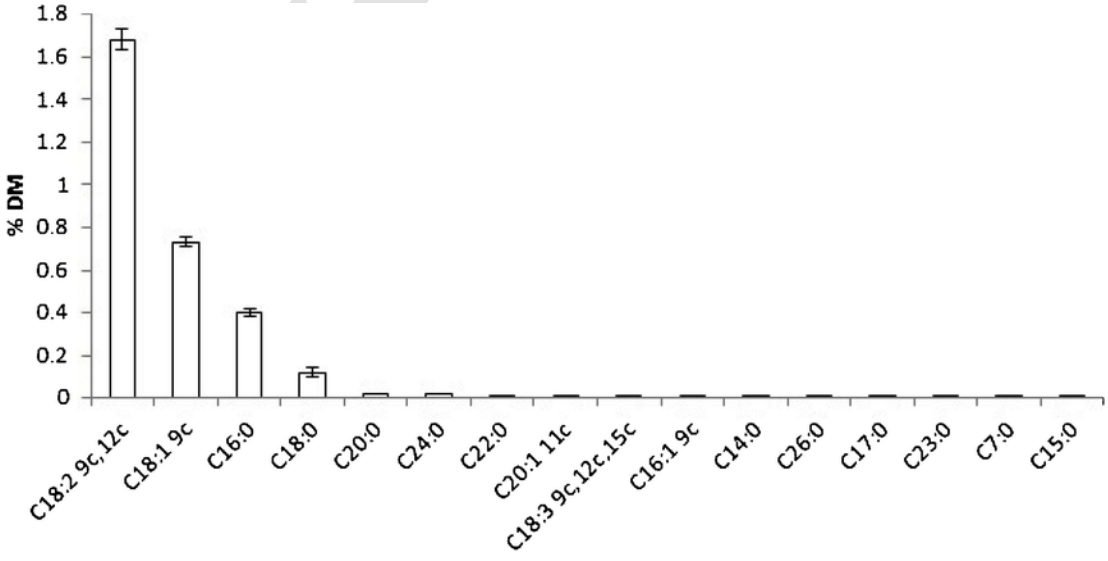


**Fig. 2.** Fatty acid profile of cardoon press cake (Vertical bars represent standard deviations).

**Table 2**
Content of total phenolics, non-tannic and tannic phenolics, abundance of phenolic sub-classes (following untargeted profiling), and PPO activity in cardoon press cake (average ± standard deviation).

| Items | Units of measurements | Average values |
|---|---|---|
| Total phenolics | mg TAE $g^{-1}$ | 32.7 + 0.02 |
| Non tannic phenolics | mg TAE $g^{-1}$ | 17.2 + 0.03 |
| Tannic phenolics | mg TAE $g^{-1}$ | 15.4 + 0.01 |
| Phenolic acids | mg /100 $g^{-}$ ferulic acid eq. | 9.99 + 2.56 |
| Tyrosols and hydroxybenzaldehydes | mg /100 g tyrosol eq. | 7.52 + 0.02 |
| Alkylphenols | mg /100 g 5-pentadecylresorcinol eq. | 6.66 + 0.41 |
| Flavones | mg /100 g luteolin eq. | 5.46 + 0.41 |
| Dibenzylbutyrolactones | mg /100 g matairesinol eq. | 3.65 + 0.74 |
| Furofurans | mg /100 g sesamin eq. | 1.20 + 0.37 |
| Stilbenes | mg /100 g resveratrol eq. | 0.55 + 0.02 |
| Anthocyanins | mg /100 g cyanidin eq. | 0.06 + 0.01 |
| Total PPO activity | μKatal $g^{-1}$ DM | 9.01 + 0.83 |

TAE = tannic acid equivalent

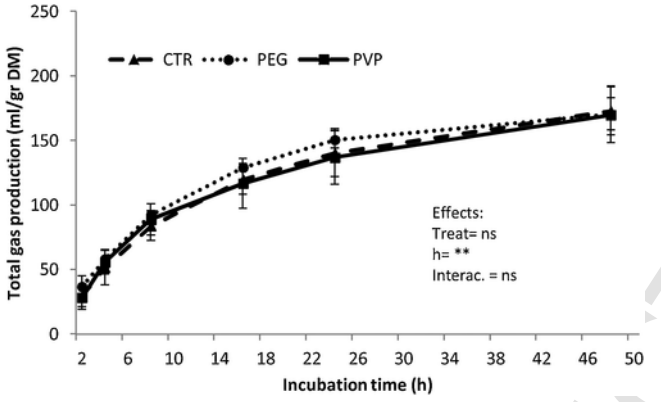


**Fig. 3.** Effects of antitannic agent (PEG or PVP) on *in vitro* total gas production (ml/g DM) of cardoon press cake incubated with ruminal liquid at different time (h) of rumen incubation. * = P ≤ 0.05; ** = P ≤ 0.01; ns = not statistically significant (n = 54); (Vertical bars represent standard errors of means).

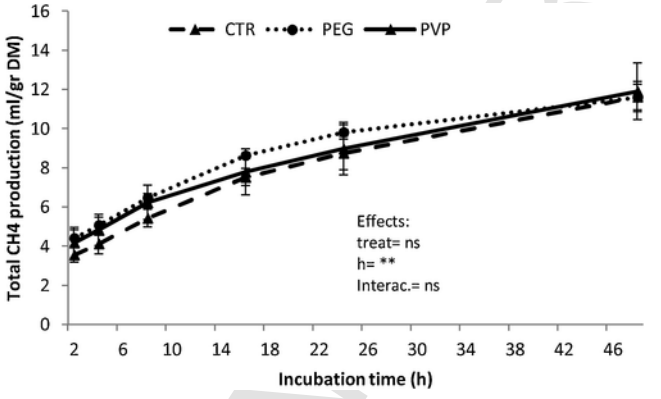


**Fig. 4.** Effects of antitannic agent (PEG or PVP) on *in vitro* methane production (ml/g DM) of cardoon press cake incubated with ruminal liquid at different time (h) of rumen incubation. * = P ≤ 0.05; ** = P ≤ 0.01; ns = not statistically significant (n = 54); (Vertical bars represent standard errors of means).

and lipid digestion at ruminal level as well as their effects in terms of gas and methane production it is necessary to quantify and characterize their contents in cardoon press cake.

The assumptions of our study were based on previous results from Decandia et al. (2008), which have found a significant effect of phenolic compounds (likewise tannins) on crude protein utilization when using an anti-tannic agent (polyethylene glycol or PEG) with a ratio between PEG and tannic phenolics of 0.37:1. On the other hand, Besharati and Taghizadeh (2011) have reported that an addition of polyvinylpyrrolidone (PVP) 25,000 or PEG 6000 (ratio PEG-PVP/tannic phenols = 26:1) as a supplement of different byproducts reduce the adverse effect of tannins on nutrient availability in ruminants.

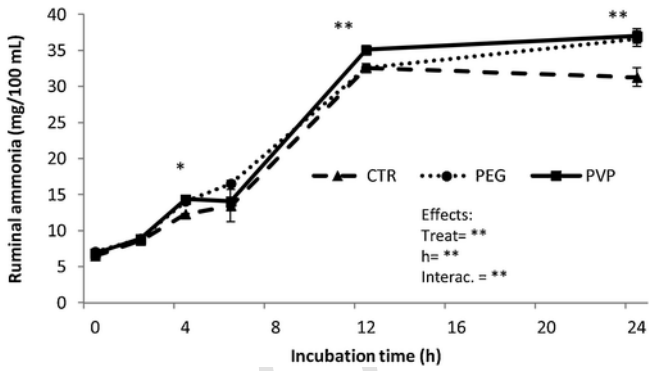


**Fig. 5.** Effects of antitannic agent (PEG or PVP) on *in vitro* ammonia level (mg/100 ml) of cardoon seed press cake incubated with ruminal liquid at different time (h) of rumen incubation. * = P ≤ 0.05; ** = P ≤ 0.01; ns = not statistically significant (n = 54); (Vertical bars represent standard errors of means).

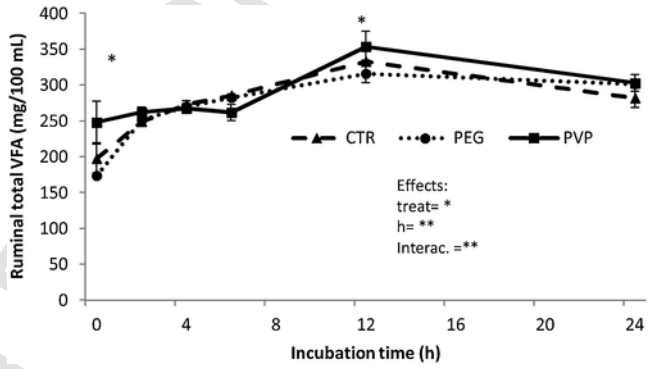


**Fig. 6.** Effects of antitannic agent (PEG or PVP) on *in vitro* VFA (mg/100 ml) of cardoon seed press cake incubated with ruminal liquid at different time (h) of rumen incubation. * = P ≤ 0.05; ** = P ≤ 0.01; ns = not statistically significant (n = 54); (Vertical bars represent standard errors of means).

In particular, phenolics compounds, other plant secondary metabolites (Buccioni et al., 2012) and the polyphenol oxidase (PPO) enzymatic group contained in the cardoon seeds could interfere with ruminal dynamics of the fibrous and nitrogen fractions and fatty acid biohydrogenation (Lee, 2014).

Overall, this research was addressed to study feeding value and traits of the cardoon press cake, supplied as a biorefinery byproduct from an agro-industrial high-value chain, to be used as a novel supplement for feeding small ruminants reared in the same Mediterranean area.

Specific aims of this work were:

i) determine cardoon press cake nutritional composition, amino acid profile, fatty acid profile, phenolic compounds, and PPO activity.
ii) evaluate the effect of anti-tannic agents, by testing the cardoon press cake with an *in vitro* fermentation, on total gas and methane productions, ammonia level, ruminal volatile fatty acids (VFA) profile, ruminal biohydrogenation of linoleic and linolenic acids, crude protein degradation and fiber digestion.

**Table 3**
Effects of antitannic agent (PEG or PVP) on VFA profile of cardoon press cake incubated with ruminal liquid at different time (h) of rumen incubation.

| | time of rumen incubation Hrs | CTR | PEG | PVP | Sem | Treat | H | Interac. |
|---|---|---|---|---|---|---|---|---|
| Acetate (%) | 0 | 61.91 | 62.70 | 60.64 | 0.51 | ns | ** | ns |
| | 2 | 60.17 | 60.27 | 61.53 | 0.44 | | | |
| | 4 | 58.82 | 59.23 | 59.00 | 0.19 | | | |
| | 6 | 58.81 | 58.64 | 58.51 | 0.08 | | | |
| | 12 | 56.01 | 55.68 | 55.77 | 0.08 | | | |
| | 24 | 55.90 | 55.69 | 55.90 | 0.06 | | | |
| Propionate (%) | 0 | 21.99 | 21.52 | 21.48 | 0.20 | ns | ** | ns |
| | 2 | 23.30 | 23.34 | 22.67 | 0.27 | | | |
| | 4 | 23.21 | 23.45 | 23.66 | 0.07 | | | |
| | 6 | 23.44 | 23.52 | 23.46 | 0.04 | | | |
| | 12 | 22.94 | 23.14 | 22.98 | 0.06 | | | |
| | 24 | 22.67 | 22.78 | 22.72 | 0.03 | | | |
| Butyrate (%) | 0 | 12.88 | 12.78 | 12.73 | 0.11 | ns | ** | ns |
| | 2 | 13.53 | 13.48 | 13.04 | 0.16 | | | |
| | 4 | 13.86 | 13.74 | 13.78 | 0.05 | | | |
| | 6 | 13.86 | 13.92 | 13.76 | 0.03 | | | |
| | 12 | 14.10 | 14.19 | 14.08 | 0.03 | | | |
| | 24 | 14.08 | 14.19 | 14.12 | 0.04 | | | |
| Total isoacids (%) | 0 | 3.22 | 3.00 | 5.15 | 0.75 | ns | ** | ns |
| | 2 | 3.00 | 2.92 | 2.75 | 0.06 | | | |
| | 4 | 4.11 | 3.58 | 3.56 | 0.20 | | | |
| | 6 | 3.89 | 3.92 | 4.28 | 0.08 | | | |
| | 12 | 6.96 | 7.00 | 7.17 | 0.04 | | | |
| | 24 | 7.35 | 7.34 | 7.26 | 0.04 | | | |
| C2/C3 ratio | 0 | 2.82 | 2.91 | 2.82 | 0.02 | ns | ** | ns |
| | 2 | 2.58 | 2.58 | 2.73 | 0.05 | | | |
| | 4 | 2.53 | 2.53 | 2.49 | 0.01 | | | |
| | 6 | 2.51 | 2.49 | 2.49 | 0.01 | | | |
| | 12 | 2.44 | 2.41 | 2.43 | 0.01 | | | |
| | 24 | 2.47 | 2.45 | 2.46 | 0.01 | | | |
| (C2 + C4)/C3 ratio | 0 | 3.40 | 3.51 | 3.42 | 0.02 | ns | ** | ns |
| | 2 | 3.16 | 3.16 | 3.30 | 0.05 | | | |
| | 4 | 3.13 | 3.11 | 3.08 | 0.01 | | | |
| | 6 | 3.10 | 3.09 | 3.08 | 0.01 | | | |
| | 12 | 3.06 | 3.02 | 3.04 | 0.01 | | | |
| | 24 | 3.09 | 3.07 | 3.08 | 0.01 | | | |

SEM = standard error of means; * = $P < 0.05$; ** = $P < 0.01$; ns = not statistically significant.

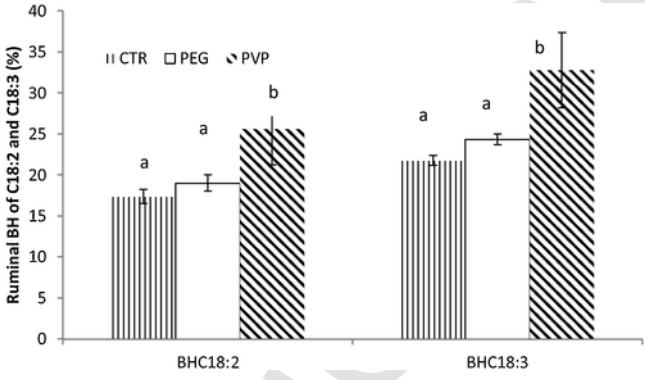


**Fig. 7.** Effects of antitannic agent (PEG or PVP) on ruminal biohydrogenation of linoleic (BHC18:2) and linolenic (BHC18:3) acids of cardoon press cake incubated 6 h with ruminal liquid. Different letters represent the effect of different treatment (a, b differ at least at $P \leq 0.05$) (n = 18). (Vertical bars represent standard errors of means).

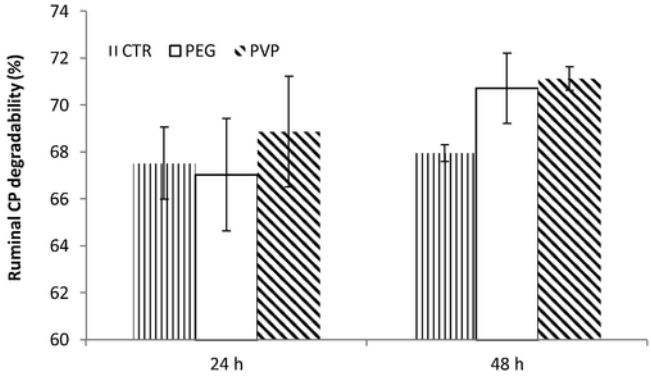


**Fig. 8.** Effects of antitannic agent (PEG or PVP) on ruminal CP degradability (% CP) of cardoon seed press cake incubated at 24 and 48 h with ruminal liquid (n = 27); (Vertical bars represent standard errors of means).

## 2. Materials and methods

The study was conducted in 2017, at the facilities of Bonassai (Olmedo) research station located in north western Sardinia, Italy (40 °N, 8 °E, 32 m a.s.l.). All the procedures were conducted in accordance with the ethical guidelines in force at Agris, in compliance with the European Union Directive 86/609/EC and the recommendation of the Commission of the European Communities 2007/526/EC.

The investigated cardoon press cake was kindly supplied by Novamont S.P.A. (Novara, Italy) and was obtained as byproduct from cardoon seeds crushing. Harvesting of achenes took place in August 2016, using a combine equipped with an innovative flexible head for cardoon crop as described in details by Pari et al. (2016). Two months after harvesting, achenes were cold pressed for oil extraction with a mechanical screw press and the partially defatted solid remaining after pressing was the cardoon press cake.

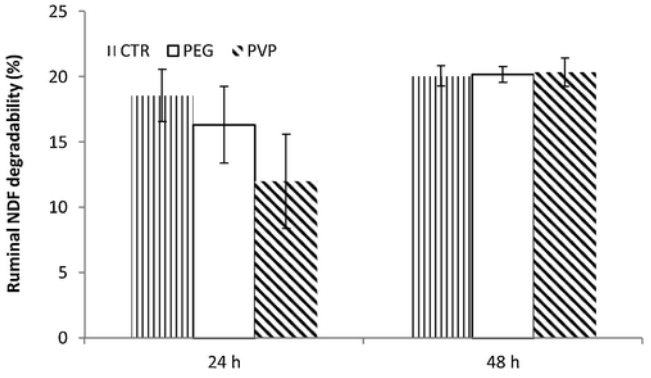


**Fig. 9.** Effects of antitannic agent (PEG or PVP) on ruminal NDF digestibility (% NDF) of cardoon seed press cake incubated at 24 and 48 h with ruminal liquid. (n = 27) (Vertical bars represent standard errors of means).

### 2.1. Press cake sample analysis

Samples of cardoon press cake were ground to a 1 mm mesh size with a Wiley mill to be further analysed. Bromatological determinations were performed in duplicate The dry matter (DM) was determined by oven drying at 105 °C to constant weight (AOAC, 2005; ref. 934.01), organic matter and total ash by muffle furnace (AOAC, 2005; ref. 942.05), crude protein (CP) by the Kjeldhal method (AOAC, 2005; ref. 976.05) and ether extract (EE) by Soxhlet analysis (AOAC, 2005; ref. 2003.05). In addition, the starch was measured according to European Commission (1999), while water-soluble carbohydrates (WSC) contents were measured according to Deriaz (1961). The NDF (neutral detergent fiber assayed with a heat stable amylase and expressed exclusive of residual ash), ADF (acid detergent fiber, expressed exclusive of residual ash) and ADL (acid-detergent lignin) were determined as described by Van Soest et al. (1991) using an Ankom 220 Fiber Analyzer equipment (Ankom Technology, New York, USA). The CP fractions, *i.e.* buffer soluble protein (BSP), non-protein nitrogen (NPN), and acid detergent insoluble nitrogen (ADIN) were analyzed according to Licitra et al. (1996).

The following analysis were all performed in triplicate. The powdered material was used for extract preparation of total phenolics as reported by Cabiddu et al. (2010). Total phenolics (TotP), non-tannic phenolics (NTP) and tannic phenolics (TP) were analysed using Folin–Ciocalteu reagent and expressed as tannic acid equivalent (mg TAE $g^{-1}$ DM) according to procedures previously described (Cabiddu et al., 2010). Briefly, the samples (0.5 g each) were incubated in a solution of acidified methanol and then filtered. The filtrate was added with a solution including Folin-Ciocalteau reagent and sodium carbonate. After two hours, the samples were assayed for total polyphenols by a spectrophotometer (765 nm) using cathechin as standard. To measure non-tannic polyphenols the same procedure was used but the filtrate was pretreated with methyl-cellulose and ammonium sulphate before adding the Folin-Ciocalteau reagent. Tanninc phenolics were measured as the difference between total and non-tannic polyphenols. The analysis of phenolic profile was carried out in agreement with Rothwell et al. (2013), Lucini et al. (2017) and Rocchetti et al. (2017). In brief, cardoon press cake samples (1 g each) were extracted in a solution of 15 ml of 1% formic acid in 80:20 methanol/water solution (LCMS grade, VWR, Milan, Italy) at room temperature using an Ultra-Turrax apparatus. Phenolic compounds were then screened in the extracts through UHPLC liquid chromatography (Agilent 1290 series) coupled to a quadrupole-time-of-flight mass spectrometer (G6550 iFunnel), (Lucini et al., 2017). Deconvolution and compounds annotation were carried out using the software Profinder B.06 (from Agilent Technologies), based on the database exported from Phenol-Explorer 3.6 (Rothwell et al., 2013). Both monoisotopic accurate mass and isotopic profile (*i.e.*, isotope spacing and ratio), together with 5 ppm tolerance for mass accuracy, were used for identification. Identification was carried out according to Level 2 (putatively annotated compounds), as set out by the COSMOS Metabolomics Standards Initiative (http://cosmos-fp7.eu/msi).

To gain semi-quantitative data, calibration curves were prepared from standard solutions of single pure phenolics (Extrasynthese, Lyon, France), as previously reported (Rocchetti et al., 2017). Ferulic acid (for hydroxycinnamic acids and other phenolic acids), matairesinol (for dibenzylbutyrolactone and dihydroxydibenzylbutane lignans), sesamin (furan and furofuran lignans), cyanidin (anthocyanins), catechin (flavanols), luteolin (flavones and other remaining flavonoids), resveratrol (stilbenes), 5-pentadecylresorcinol (alkylphenols) and tyrosol (tyrosols and other remaining phenolics) were used with this purpose.

For PPO activity assay, samples (1 g each) were extracted according to the method of Winters and Minchin (2001) and assayed according to Robert et al. (1995) with some modifications as reported by Cabiddu et al. (2010). Amino acids analysis (1 g each sample) was performed using the Carlo Erba model 3A29 amino acid analyzer (Carlo Erba Strumentazione, Corsico, Italy) (Moore et al., 1980). A total of 27 samples were analyzed (3 from cardoon press cake and 24 from *in vitro* fermentations) for fatty acid profile. Lipid was extracted as reported by Cabiddu et al. (2010). The extract was then dried under $N_2$ at 50 °C, resuspended in 1 ml of heptane and converted to fatty acid methyl esters (FAME), using the bimethylation procedure (1.4 mol $L^{-1}$ HCl in methanol and 0.5 mol $L^{-1}$ NaOH in methanol) described by Kramer and Zhou (2001) to give the total fatty acid content, used to calculate biohydrogenation of the C18 poly unsaturated fatty acids (PUFA). FAME were analysed by gas chromatography on a CP Sil 88 FAME column (100 m × 0.25 mm i.d., Chrompack UK Ltd, London, UK) with split injection (30:1). Peaks were identified from standards (ME61, Larodan Fine Chemicals, Malmo, Sweden; S37, Supelco, Poole, UK) and quantified using the internal standard (C21:0) for total fatty acid analyses.

### 2.2. In vitro fermentation experimental design

Based on the studies of Decandia et al. (2008) and Besharati and Taghizadeh (2011), different tannin binding agents were compared against a control (press cake without anti-tannic agent). To evaluate the effect of phenolic compounds on protein, fiber and lipid utilization at ruminal level, we used the ratio (0.37 w/w) between PEG4000 or PVP25000/tannic phenolic already tested by the same authors, The following treatments were thus compared: cardoon press cake alone (CTR), cardoon press cake + 2 mg of PEG4000 (PEG) or + 2 mg of PVP25000 (PVP).

For *in vitro* test, rumen liquor was provided by a slaughterhouse from culled dairy sheep (4 sheep per rumen inoculum) and was delivered to the laboratory within half an hour in airtight glass-bottles filled with $CO_2$ and immersed in thermoses filled with 39 °C water. The rumen fluid was strained through 3 layers of cheesecloth to eliminate feed particles (this pore size allows the passage of the protozoa population during the straining) and kept at 39 °C under constant infusion of $CO_2$ to maintain anaerobic conditions. The time required for all operations was < 10 min.

#### 2.2.1. Assessment of gas and methane production

Fermentation was conducted as following: 440 ± 0.2 mg of freeze dried samples were placed in each 120 ml serum bottle pre-warmed at 39 °C and flushed with $CO_2$. In a series of 12 bottles, 20 ml of rumen fluid (strained rumen fluid) and 40 ml of medium (Menke and Steingass, 1988) were added, then the bottles were sealed hermetically with butyl rubber stopper and aluminium crimp seals. Blanks with only

buffered rumen fluid were also incubated. Samples were incubated in three replicates for each treatment.

The gas production was recorded at 2, 4, 8, 16, 24 and 48 h, respectively, using the pressure transducer technique, as described by Theodorou et al. (1994). At the same time, gas samples were taken from the headspace of the serum bottles for determination of methane concentration. After 48 h, the fermentation was stopped cooling the bottles. Total gas and methane production were expressed as mL $g^{-1}$ DM incubated. Methane analysis was determined by gas chromatography using a Dany Master Fast GC with a column Valcoplot VP Allumina $Na_2SO_4$ 30 mt Ǿ 0.53 mm.

*2.2.2. Assessment of ruminal volatile fatty acids (VFA) and ammonia*

The procedure for rumen liquid preparation, medium ingredients, rumen liquid/medium ratio and incubation was similar as above. A total of 54 bottles were incubated in a shaking water bath at 39 °C for 0, 2, 4, 6, 12 and 24 h in three replicates per treatment. At each sampling time, the corresponding incubated bottles were takeout and stored at −20 °C till VFA and ammonia analysis. Samples of buffered rumen fluid were also taken at time 0 to determine net production of VFA and $NH_3$-N. VFA profiles were expressed as % of total VFA, whereas $NH_4$ and total VFA were expressed as mg/100 ml of rumen fermentation liquid.

*2.2.3. Assessment of PUFA biohydrogenation*

Each treatment was incubated in triplicate in rumen inoculum at 39 °C, over two time points (0 and 6 h). Rumen inoculum was processed according to Cabiddu et al. (2010). Samples of each treatment (2060.0 ± 0.48 mg DM) were weighed into 24 tubes, with six tubes allocated to each treatment (CTR, PEG and PVP), three tubes per time point (0 and 6 h); a series of 6 tubes as blanks (rumen inoculum + buffer) without samples were also incubated. The biohydrogenation of linoleic (C18:2) and linolenic (C18:3) acids were determined by subtracting the initial value in the culture (time zero) to the value at the end of fermentation as reported by Cabiddu et al. (2010).

*2.2.4. Assessment of CP degradability and NDF digestibility*

Fermentations were conducted as described by Doane et al. (1997) and modified by Ross et al. (2013). Total bottles incubated were 27 400 ± 0.2 mg of DM samples (CTR, PEG and PVP treatments) were weighted in each bottle and incubated with inoculum having rumen liquid:medium ratio of ¼ for 24 and 48 h. The anti-tannic agents were weighted individually for each bottle (2 mg), except for CTR. In brief, the digestibility of NDF (RDNDF) and degradation of CP (RUDCP) were determined by their differences before incubation and after 24 and 48 h and their values were corrected from microbial contamination in agreement with Doane et al. (1997) and Ross et al. (2013).

*2.3. Data analyses*

Untargeted phenolic profiling data were aligned, then filtered by abundance (abundance > 10,000 counts) and by frequency (only those compounds being present in 100% of replications were retained) using Profinder B.06 (Agilent technologies), prior to quantification (Lucini et al., 2016).

Considering 3 anti-tannic treatments (PEG, PVP and CTR), 3 replications for each and related sampling times (ST), the total number of samples analysed were: 54 (from 6 ST) for each gas and $CH_4$ productions, 54 (6 ST) for each VFA and NH3, 27 (3 ST) for each ruminal NDF digestibility and CP degradability, and 18 (2 ST) for ruminal biohydrohenation of linoleic and linolenic acids, respectively. Laboratory measurement values were subjected to a two-way analysis of variance in agreement to a completely randomized design by using GLM procedure of SAS (2003) to test the effects of anti-tannic treatments, sampling time and their interaction, considering the treatment and sampling time as fixed effects. A difference between treatments was declared significant when $P < 0.05$ using the F test.

## 3. Results and discussion

*3.1. Chemical composition of cardoon press cake*

Regarding nutritional profile, results (Table 1) indicated a moderate content of CP and EE according to Genovese et al. (2016), whereas the content of fiber fractions (NDF, ADF and ADL) were higher compared to Cajarville et al. (2000) with particular reference to ADF and lignin (+30%). Compared to the press cake from sunflower (*Helianthus annuus* L.), which belongs to the same botanical family (Asteraceae), cardoon press cake showed lower content of CP (−37%), and higher contents of NDF (+24%), ADF (+36%) and ADL (+30%) (NRC, 2001). The high level of ADF and ADL can negatively affect the digestibility of CP and fiber; in fact, the level of ADIN is near 20%, which means that a remarkable amount of protein is not available at ruminal/gastrointestinal tract level (Chrenkova et al., 2014). In terms of carbohydrates storage, cardoon press cake represented a very scanty source of starch with a value of 1.22% on DM basis (Table 1). Protein fractions were mainly (59%, Table 1) represented by soluble proteins, rapidly available at ruminal level, whereas 36% of CP were classified (NDIN, ADIN) as slowly degradable (Pichard and Van Soest, 1977). In terms of protein fraction, cardoon press cake presents a CP profile less available at ruminal level since the content of ADIN is 60% higher than sunflower cake (NRC, 2001).

Regard to amino acid profile (Fig. 1), the essential amino acid groups (Boisen et al., 2000), (Thr, Met, Ile, Leu, Val, Phe, His, Lys, Tyr) were well represented, except for tryptophan (Trp), according to Genovese et al. (2016) findings. Although current knowledge in requirements of individual amino acids in ruminant nutrition is too limited (cows and sheep) (Lapierre et al., 2016), cardoon press cake represents as a valuable source of essential amino acids such as Val, Phe, Lys, Thr, and His in comparison with sunflower cake (NRC, 2001). The main representative fatty acids were linoleic and oleic acids with 1.7 and 0.7% on DM basis, respectively (Fig. 2), in accordance with Genovese et al. (2016). This could be of interest with regard to the healthiness of dairy products; in fact, linoleic acid is a precursor of unsaturated fatty acid in dairy products (Buccioni et al., 2012) as well as of arachidonic acid (AA), which is incorporated into cell and cell membranes providing a structural support. In addition, AA is a precursor of prostaglandins 2 series (PGI2) and thromboxanes, leucotrienes and other lipoxygenase/cyclooxygenase, which play an important role in the inflammatory processes. It is well known that most of the mediators formed from eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) (ω-3) are anti-inflammatory, whereas those formed from AA (ω-6) are pro-inflammatory or show other disease-propagating effects (Schmitz and Ecker, 2008). Cardoon press cake, compared to sunflower cake showed higher proportion of linoleic acid (+40%), palmitic acid (+220%) and lower oleic acid (−46%). A higher content of linoleic acid in animal diet could improve animal fertility (Rodney et al., 2015) and dairy products healthiness (Buccioni et al., 2012).

Based on previous studies (Cajarville et al., 2000; Irshaid et al., 2003; Correddu et al., 2015) dealing with the potential replacing value of different byproducts on animal supplementation and according to Cannas et al. (2006), it could be assumed that an inclusion of 250 g of cardoon press cake might replace 100 g of soybean or 150 g of sunflower cakes, in dairy sheep grazing ryegrass. The proposed cardoon cake supplementation could support a milk production of 2.5 kg per ewe and per day, reducing feeding costs without negative effects on animal performances. Among plant secondary metabolites, phenolic compounds were widely represented, with 1:1 ratio between NTP and TP (Table 2). To date, very limited information is available regarding the

plant secondary metabolites content and composition of cardoon press cake. Genovese et al. (2016) have reported a total phenolic value of 12 mg $g^{-1}$ DM of gallic acid equivalent in cardoon press cake, which was almost three times lower than our values; however, they have adopted a different method and solvent for extraction. Comparable levels of total phenolics have been reported by Falleh et al. (2008) in cardoon seeds and by Molle et al. (2008) and Sulas et al. (2017) in forages species likewise chicory (*Cichorium intybus* L.), subterranean clover (*Trifolium subterraneum* L.), and garland (*Chrysanthemum coronarium* L.). It is known that even moderate levels of phenolics could affect the *in vitro* DM digestibility. A recent paper has suggested that different phenolics could interact with ruminal microorganisms (Guerra-Rivas et al., 2017), resulting in a reduction in DM and OM digestibility with no clear relationship to the kind of phenolic profile. For example, higher level of anthocyanins in grape pomace seems to increase the *in vitro* DM digestion (Guerra-Rivas et al., 2017). These opposite effects could be due to the different phenolic profile detected in different byproducts. In our study, the highest level in phenolic profile was recorded for phenolic acids (PA), followed by tyrosols and hydroxybenzaldehydes (THB), alkylphenols (APH) and flavones (FL), as reported in Table 2. Lignans (both dibenzylbutyrolactones and furofurans) were lower but still present, whereas very limited amounts of stilbenes and anthocyanins have been detected. Interestingly, Oskoueian et al. (2013) reported that flavones are potent inhibitors of rumen microbial ecosystem compared to other phenolics, as confirmed also by Tim Cushnie and Lamb (2005) and suggesting a potential influence on the nutrients utilization in ruminants.

The enzyme polyphenol oxidase was also detected (Table 2). To our knowledge, the detection of PPO in cardoon press cake has never been reported; our results showed a moderate activity of PPO, which is 30% less compared to other plants rich in PPO content like red clover (*Trifolium pretense* L.). PPO is a copper metalloprotein, which functions in the presence of oxygen, and is able to reduce proteolysis and lipolysis (at ruminal level or during silage making) through deactivation of proteolytic/lipolytic enzymes and/or through formation of protein–phenol–lipid complexes (Lee, 2014). The occurrence of PPO in forages has improved protein and lipid efficiency by 15–20% (Buccioni et al., 2012).

### 3.2. Effects of anti-tannic treatments on in vitro fermentation

Total gas and methane productions were affected by incubation time (h: $P < 0.01$), while anti-tannic treatment and the interaction between treatment x time were not significant (Fig. 3 and 4). Comparing with Lee et al. (2003), cardoon press cake produces less total gas and methane $g^{-1}$ DM incubated than that obtained using corn (*Zea mays* L.) germ meal as substrate. This could be due to an associative effect between different phenolic compounds and PPO, which occurs in cardoon cake according to Cieslak et al. (2013). Based on the bibliography data, ruminants emit gases (likewise $CH_4$ namely also greenhouse gases) to the atmosphere from animal feed digestion (rumen and enteric fermentation). Methane is a short-lived climate pollutant with a global warming potential 36 times higher than that of carbon dioxide for the 12 years it lasts in the atmosphere. In addition, there is evidence of the effect of phenolic compounds on the reduction (12 to 46%) of methane emission from livestock production, as reported by Knapp et al. (2014). For these reasons, an increased knowledge in plant secondary metabolites interaction with methane production is a new challenge to mitigate gas emission from livestock. Ruminal ammonia level and total VFA ($P < 0.01$) were influenced by treatment and time (Fig. 5 and 6). As expected, interaction between anti-tannic treatment and incubation time was significant for both ammonia and total VFA (Figs. 5 and 6, $P < 0.01$). PEG and PVP treatments increased the level of ruminal ammonia at 24 h compared to CTR (Fig. 5, $P < 0.05$). Ruminal fermentation parameters (in terms of acetate, propionate, butyrate and total isoacid proportions) were not influenced by treatment, whereas sampling time, as expected, affected significantly ($P < 0.01$) their proportion, with minimum content at 0 h and maximum level at 24 h (Table 3). Similarly, C2/C3 and (C2 + C4)/C3 ratios were influenced by sampling time ($P < 0.01$) with highest value at 0 h (Table 3). PVP tended to increase linoleic and linolenic acid biohydrogenation (+47% and +55% respectively, $P < 0.06$) compared to CTR, without any effect from PEG treatment (Fig. 7). These effects did not totally agree with Cabiddu et al. (2009), which found an increase in linolenic acid (less biohydrogenation) in milk produced from sheep grazing sulla (*Sulla coronaria* L.) (4% of condensed tannin) supplemented with PEG 4000 at a similar anti-tannic/tannin ratio. Our results suggest an increase of total availability of linoleic acid in cardoon press cake, since phenolics and PPO could improve the transfer efficiency ratio of PUFA from feed to animal products. Ruminal NDF and CP degradability corrected in terms of microbial contamination were measured according to Doane et al. (1997). No effect of treatments were detected both for RUDCP and RDNDF (Figs. 8 and 9), probably due to the higher level of ADIN that was largely undegradable. In fact, the $NH_4$ level in ruminal liquid is more sensitive to the anti-tannic effect since soluble fraction of protein is more available than ADIN (Pichard and Van Soest, 1977). To our knowledge, this is the first paper that focusses on *in vitro* ruminal digestion of cardoon press cake. Overall, gas and methane production appear not influenced from phenolic compounds, likewise ammonia and VFA production. An effect of plant secondary metabolites was detected on ruminal biohydrogenation of linoleic and linolenic acids as a probably effect from PPO and phenolic compounds; on the contrary, no effect was detected for CP and NDF digestion.

## 4. Conclusions

Based on the *in vitro* test, cardoon press cake byproduct proved to be suitable as an animal feeding supplement. The composition and contents of plant secondary metabolites did not affect its nutritional value in terms of protein and fiber utilization with positive effect on lowering PUFA biohydrogenation.

The high level of linoleic acid and the low biohydrogenation ratio suggest cardoon press cake to be a good supplement of unsaturated fatty acid in ruminant nutrition to improve animal performance (better fertility) and products healthiness. In addition, the peculiar content of phenolics associated to PUFA precursors could be very useful to be transferred into dairy products. Future studies should be performed *in vivo,* to better understand the transfer of phenolics and unsaturated fatty acids in milk and dairy products, and possibly to value this byproduct with particular reference to nutraceutical and sustainability aspects. Our research contributing to the valorization of cardoon byproducts might potentially improve the economic and environmental sustainability of agro-industrial cardoon chains.

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