CYNEXT

A NEW GENERATION OF EXTRACTS FROM CARDOON FLOWERS USING PULSED ELECTRIC FIELDS (*Cynara cardunculus* L.)

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INTRODUCTION

CYNEXT

- Cardoon flower (*Cynara cardunculus* L.) (Fig.1) is a mandatory ingredient for the coagulation of a set of PDO cheeses whose action results from the biochemical activity of distinct cardosins over the milk caseins.
- CYNEXT aims a production of extracts from cardoon flower with a standardized biochemical composition and microbiological safety for the coagulation process of sheep cheese with PDO requirements.

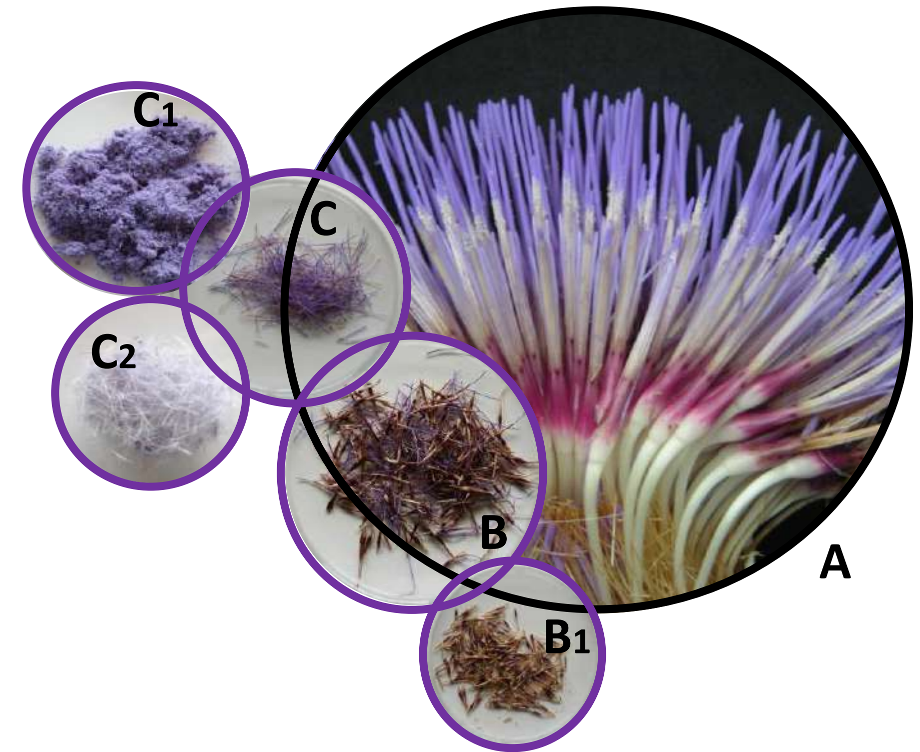

Figure 1 - (A) Cardoon inflorescence; (B) Cardoon flowers; (B1) Corollas; (C) Stigmas; (C1) Purple piderme papilosa; (C2) Inner fiber tissue.

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MORPHOLOGY AND ULTRASTRUCTURE

- For the success of Pulse Electric Fields (PEF) Technology it is essential to know the morphology and ultrastructure of the cardoon flower and the locations of cardosins (Fig.2A).

- The presence of cardosins in distinct organelles and even in the extracellular matrix (Fig.2B,C) is also a challenge to evaluate the potential of PEF technology to obtain extracts with a selective composition of distinct cardosins.

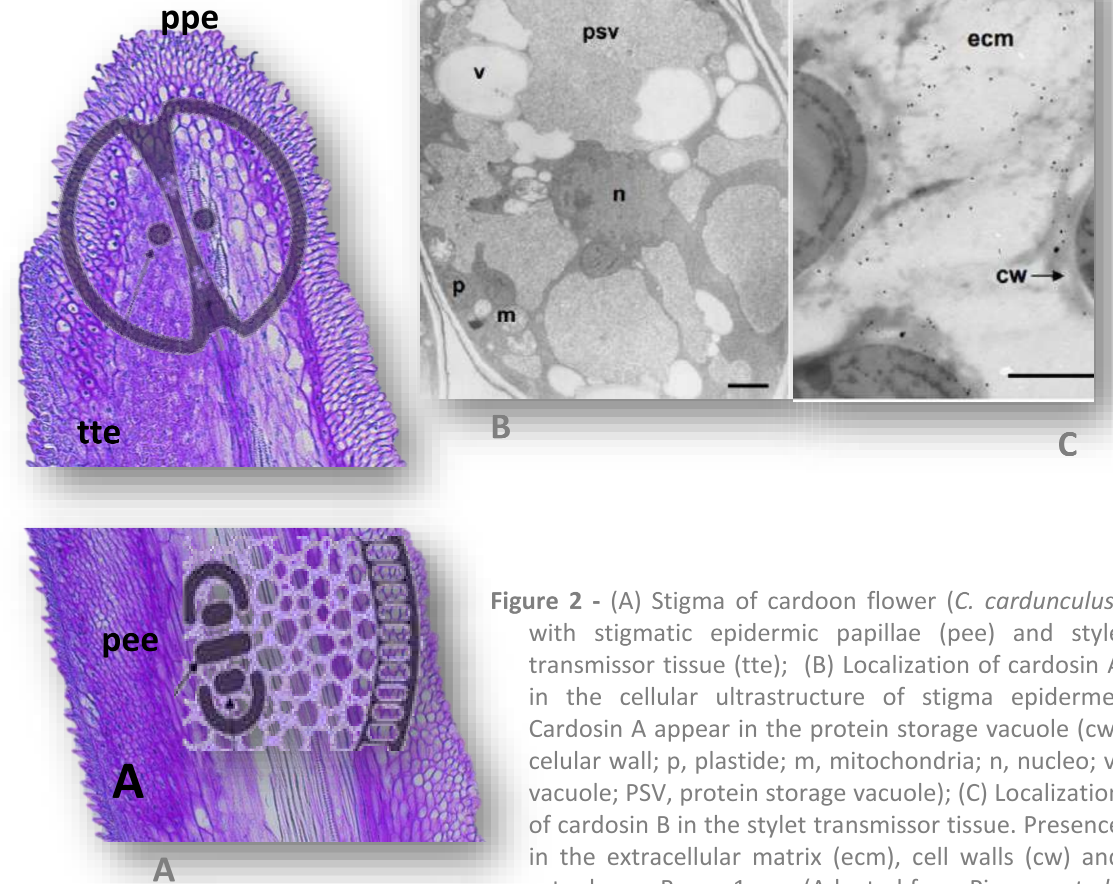

Figure 2 - (A) Stigma of cardoon flower (*C. cardunculus)* with stigmatic epidermic papillae (pee) and style transmissor tissue (tte); (B) Localization of cardosin A in the cellular ultrastructure of stigma epiderme. Cardosin A appear in the protein storage vacuole (cw, celular wall; p, plastide; m, mitochondria; n, nucleo; v, vacuole; PSV, protein storage vacuole); (C) Localization of cardosin B in the stylet transmissor tissue. Presence in the extracellular matrix (ecm), cell walls (cw) and cytoplasm. Barra: 1 µm. (Adapted from Pissarra *et al.*, 2007)

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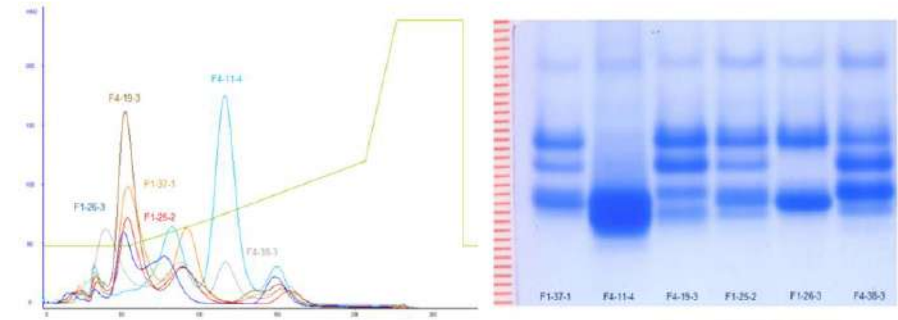
INTRODUCTION

PROTEIN DIVERSITY

- Cardoon flowers presents an extensive biochemical diversity of cardosins (A, A0 and B) (Fig.3).
- For PEF technology the knowledge of biochemical diversity and concentrations of distinct cardosins in the cardoon flowers is essential.
- Cardoon flower extracts due its biochemical characteristics permits to develop distinct curd matrix and textures in the coagulation process and cheese production (Fig.4).



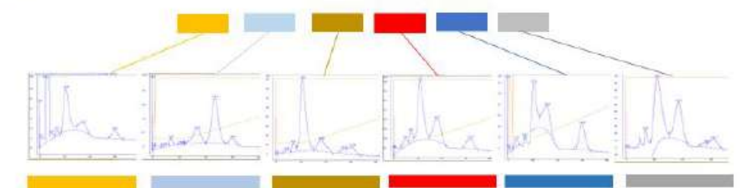


Figure 3 - Ion-Exchange cromatography and gel native of cardoon flowers from six genotypes: F1-37-1 (yellow), F4-11-4 (light blue), F4-19-3 (brown), F1-25-2 (red), F1-26-3 (dark blue), F4-38-3 (grey).

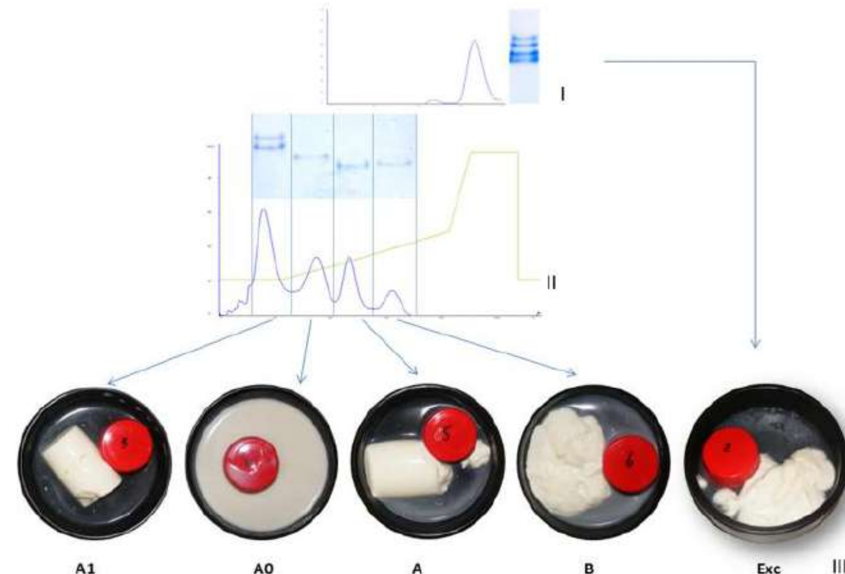


Figure 4 - Aspartic proteases biochemical diversity in milk coagulation: (I) Molecular exclusion chromatography and native electrophoresis of cardoon flower extract; (II) Ion-exchange chromatography and native electrophoresis; (III) Clotted milk using isolated aspartic proteases from the ion-exchange chromatography (A1, A0, A and B) and total aspartic proteases from the molecular exclusion chromatography (Exc).

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MATERIAL AND METHODS

CARDOON FLOWERS

- CYNEXT extracts were produced from frozen cardoon flowers submitted to the application of distinct Pulsed Electric Fields (PEF) treatments.
- The use of frozen flowers, instead of dried flowers (Fig.5A), was an innovative procedure that can optimize the extraction.
- For each treatment and replications, at a pilot scale, 20 g of flowers and 200 mL (1:10) of water (Fig.5B) were incubated before the application of PEFs conditions.

A

B

Figure 5 – (A) Frozen cardoon flowers Moisture: 41,22%
(B)Preparation of cardoon flowers for PEF technology of CYNEXT project.

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MATERIAL AND METHODS

PEF TREATMENTS

- Four PEFs treatments (A-D) with distinct number of pulses and specific energy were performed, each with three replications (Tab.1).
- The pulses were applied in a batch chamber consisting of two parallel stainless electrodes (Fig.6).

TREATMENT A: 1kV/cm; 1.8 kJ/kg; monopolar pulses of 20 µs; 10 Hz; 60 pulses

TREATMENT B: 2kV/cm; 2.4 kJ/kg; 20 µs; 10 Hz; 20 pulses

TREATMENT C: 2kV/cm; 2.4 kJ/kg; 20 µs; 10 Hz; 30 pulses

TREATMENT D: 2kV/cm; 2.4 kJ/kg; 20 µs; 10 Hz; 60 pulses

CONTROL: without PEF

		Number of Pulses			
#		**20**	**30**	**60**	**Volume**
kV/cm	**1**			A	200 mL
	2	B	C	D	200 mL

Table 1 – Number of pulses and specific energy of PEF treatments (A-D). The control were performed without application of any eletric conditions.

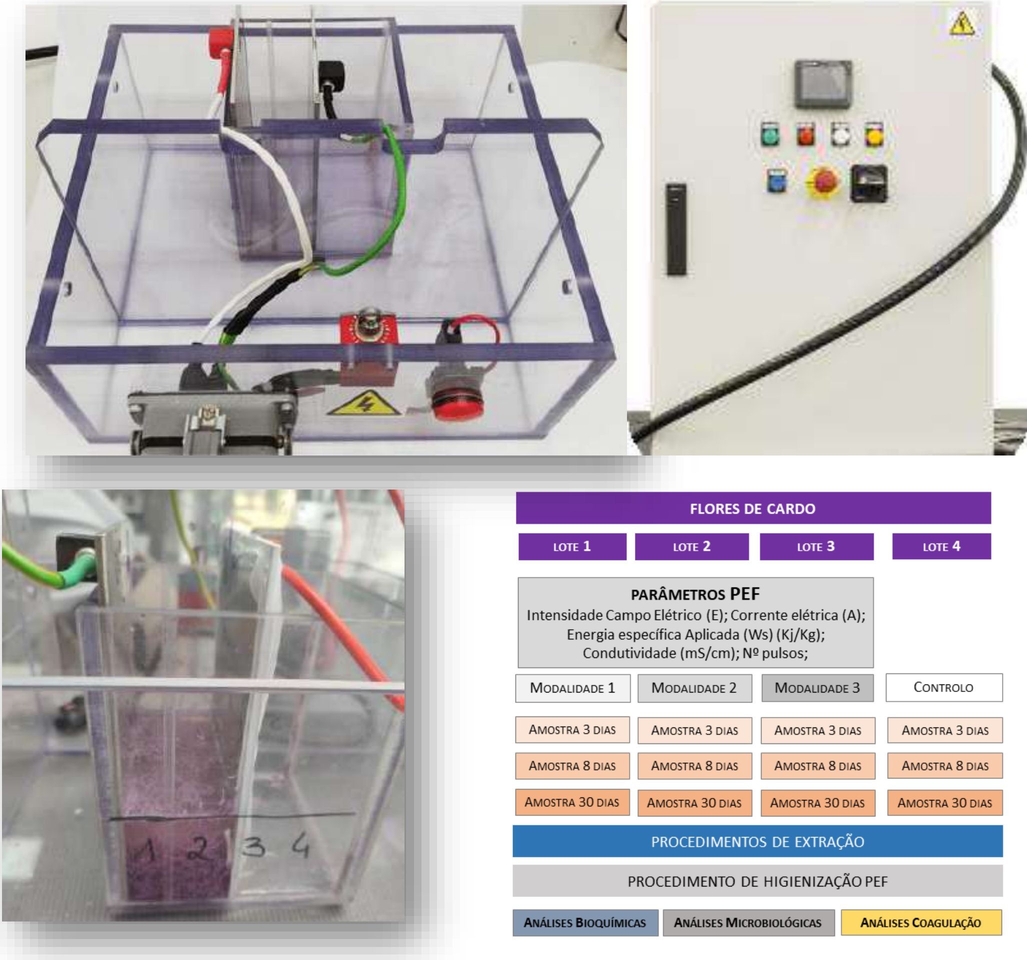

Figure 6 - Experimental methodology for PEF treatments of CYNEXT project.

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MATERIAL AND METHODS

BIOCHEMICAL CHARACTERIZATION

- The extracts were evaluated, based on biochemical markers to determine the cardosins compositions and concentrations (Fig.7).

- For biochemical characterization of PEFs extracts, without prior maceration, several analysis were performed:

  a) Spectrophotometric analysis (A260; A280 and A320)

  b) Determination of protein concentration

  c) Conductivity

  d) pH

  e) Electrophoretic and chromatographic analysis.

Figure 7 - Experimental methodology for biochemical characterization of PEF extracts

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MATERIAL AND METHODS

COAGULATION AND PROTEOLYTIC ACTIVITY

- To evaluate the potential of PEFs extracts for cheese production several assays of coagulation and proteolytic activity were performed, and the curd and whey were analyzed by NIR Technology (Buchi) (Fig.8).

  a) Moisture

  b) Dry matter

  c) Fat

  d) Protein

  e) Ash

  f) pH

Figure 8 - Experimental methodology for coagulation and proteolytic activity

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RESULTS

PH

- The pH started (T0) at 6.0 (Fig.9).

- PEFs treatments A, B and C, 24h after, presented a lower pH significantly different ($p<0.05$) compared with the control (Tab.2).

- pH seems to be an indicator to evaluate the elution of cardosins from distinct PEFs treatments due its acidic properties.

Table 2 – Evolution of pH in different PEFs treatments (A-D, control): Before PEF, 1h, 2h, 4h, 12h and 24h after PEF.

	Before PEF	1h after PEF	2h after PEF	4h after PEF	12h after PEF	24h after PEF
PEF	Média ± DP	Média ± DP	Média ± DP	Média ± DP	Média ± DP	Média ± DP
A	6.00± 0.01 ^{ab}	5.96 ± 0.02 ^{a}	5.87 ± 0.01 ^{a}	5.81 ± 0.02 ^{ab}	5.50 ± 0.06 ^{b}	5.07 ± 0.10 ^{b}
B	6.04 ± 0.05 ^{b}	5.93 ± 0.01 ^{a}	5.88 ± 0.01 ^{ab}	5.78 ±0.,06 ^{b}	5.53 ± 0.02 ^{ab}	5.10 ± 0.04 ^{b}
C	6.00 ± 0.02 ^{abc}	5.92 ± 0.01 ^{a}	5.87 ± 0.01 ^{ab}	5.81 ± 0.02 ^{ab}	5.52 ± 0.01 ^{ab}	5.08 ± 0.04 ^{b}
D	5.97 ± 0.02 ^{a}	5.91 ± 0.01 ^{b}	5.83 ± 0.02 ^{a}	5.82 ± 0.01 ^{a}	5.52 ± 0.02 ^{ab}	5.23 ± 0.04 ^{a}
Ctr	6.01 ± 0.03 ^{bc}	5.90 ± 0.02 ^{a}	5.87 ± 0.02 ^{ab}	5.82 ± 0.01 ^{a}	5.55 ± 0.01 ^{a}	5.29 ± 0.02 ^{a}

Values presented with different superscripts within columns are significantly different ($p<0.05$) by Tukey test.

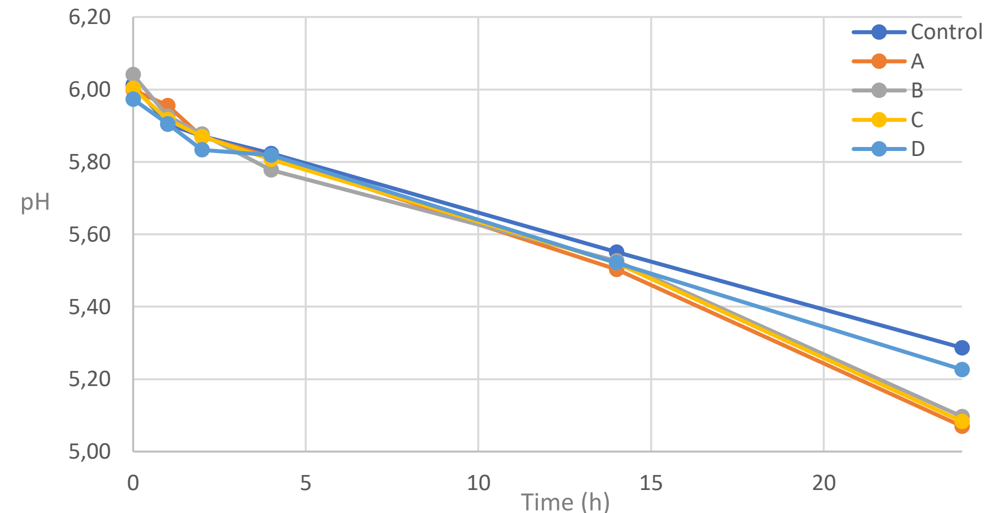

Figure 9 – Evolution of pH in different PEF treatments (A-D, CTR- control) (Before PEF, 1h after PEF, 2h, 4h, 14h, 24h and 48 h after PEF).

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RESULTS

CONDUCTIVITY

- The conductivity started (T0) at 1.4 µS/cm (Fig.10 A).
- Extract A, 1h after PEF, obtained a concentration 10% higher, compared with the control, which increased to ~20%, 2h after PEF (Fig.10 B).
- Despite extract A presented the highest value there were no significant differences (*p>0.05*) between the PEFs extracts (Tab.3).

Table 3 – Evolution of conductivity (%) in different PEFs treatments (A-D, control): 1h, 2h; 4h and 12h after PEF.

PEF	**1 h after PEF** (%) Mean ± SD	**2 h after PEF** (%) Mean ± SD	**4 h after PEF** (%) Mean ± SD	**12 h after PEF** (%) Mean ± SD
A	42.57 ± 2.17 [a]	49.26 ± 1.53 [a]	49.52 ± 2.19 [a]	52.92 ± 3.10 [a]
B	40.28 ± 5.59 [ab]	43.88 ± 5.16 [a]	45.30 ± 5.05 [a]	47.78 ± 5.28 [a]
C	37.69 ± 5.63 [ab]	42.08 ± 5.77 [a]	44.41 ± 5.86 [a]	47.21 ± 5.99 [a]
D	39.87 ± 10.51 [ab]	44.05 ± 9.79 [a]	46.10 ± 10.78 [a]	49.07 ± 11.42 [a]
Control	31.82 ± 11.51 [b]	30.15 ± 3.96 [b]	32.38 ± 3.84 [b]	41.32 ± 10.39 [b]

Values presented with different superscripts within columns are significantly different ($p<0.05$) by Tukey test.

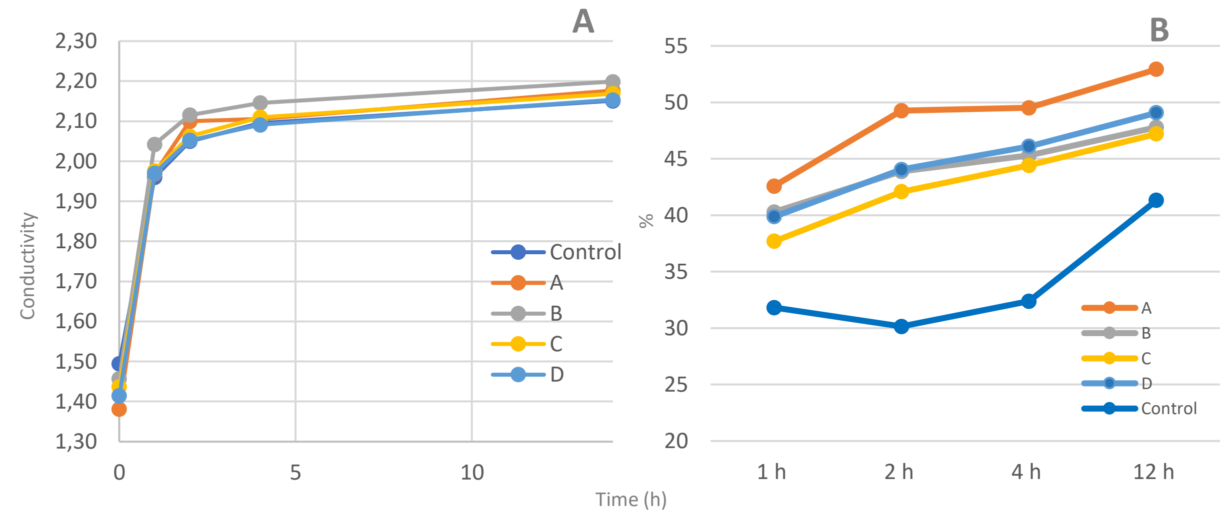

Figure 10 – Evolution of conductivity in different PEFs treatments (A-D, control) (Before PEF; 1h after PEF, 2h and 4h).

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RESULTS

PROTEIN CONCENTRATION

- The protein concentration started(T0) at 1.3 mg/ml (Fig.11A).
- Extract C, 1h after PEF, obtained a concentration 10% higher, compared with the control, which increased to 20%, 4h after PEF (Fig.11 B).
- Despite extract C presented the highest value there were no significant diferences between PEFs extracts (*p>0.05*) (Tab.4).

Table 4 – Evolution of protein concentration (%) in different PEFs treatments (A-D, control): 1h after PEF; 2h; 4h and 12h.

PEF	1 h after PEF (%) Mean ± SD	2 h after PEF (%) Mean ± SD	4 h after PEF (%) Mean ± SD	12 h after PEF (%) Mean ± SD
A	65.91 ± 11.17	107.80 ± 12.72 [a]	132.90 ± 11.91 [a]	148.60 ± 10.84 [a]
B	73.34 ± 13.36	110.12 ± 13.38 [a]	136.28 ± 13.28 [a]	152.51 ± 13.27 [a]
C	75.65 ± 5.04	115.61 ± 4.19 [a]	141.09 ± 4.78 [a]	156.51 ± 4.54 [a]
D	70.05 ± 8.64	104.63 ± 9.08 [a]	128.46 ± 10.73 [a]	143.14 ± 9.99 [a]
Control	65.24 ± 10.09	94.29 ± 1.56 [b]	119.31 ± 8.34 [b]	136.14 ± 9.51 [b]

Values presented with different superscripts within columns are significantly different ($p<0.05$) by Tukey test.

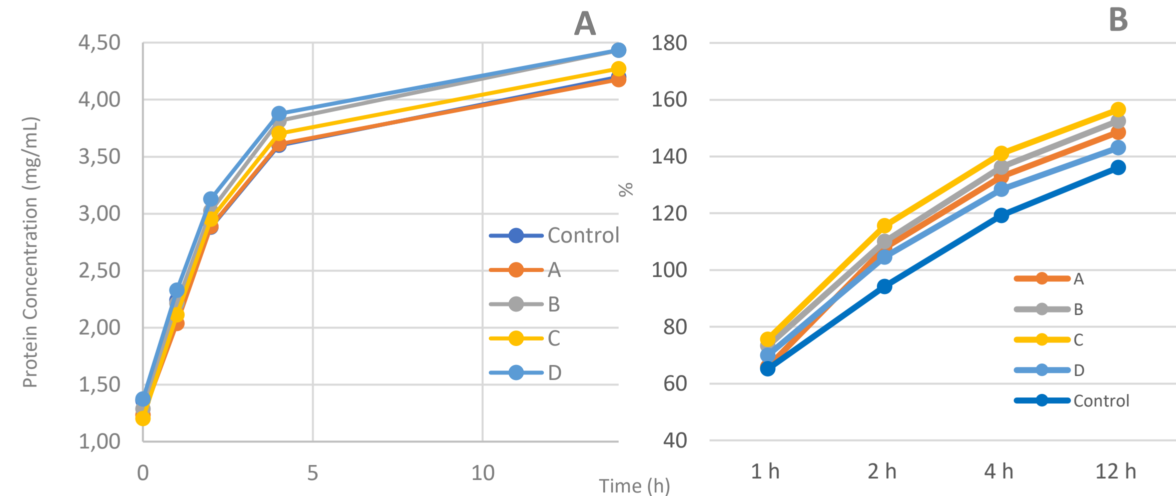

Figure 11 – Evolution of protein concentration in different PEF treatments (A-D, control) (Before PEF, 1h after PEF, 2h and 4h).

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RESULTS

A260

- The A260 started (T0) at 11 (Fig.12 A).
- Extract D, 1h after PEF treatment, obtained an absorbance 15% higher, compared with the control, which maintained till 12h (Fig.12 B).
- Despite extract D presented the highest value there were no significant differences (*p>0.05*) between the PEF extracts A, C and D (Tab.5).

Table 5 – Evolution of A260 (%) in different PEFs treatments (A-D, control): 1h after PEF; 2h; 4h e 12h.

PEF	**1 h after PEF** (%) Mean ± SD	**2 h after PEF** (%) Mean ± SD	**4 h after PEF** (%) Mean ± SD	**12 h after PEF** (%) Mean ± SD
A	68.44 ± 14.08 [ab]	80.70 ± 14.10 [a]	85.84 ± 15.13 [a]	88.42 ± 15.04 [a]
B	56.88 ± 9.59 [b]	68.29 ± 10.22 [b]	75.84 ± 10.39 [b]	78.80 ± 10.28 [b]
C	65.71 ± 0.77 [ab]	79.67 ± 2.70 [a]	87.32 ± 2.13 [a]	90.80 ± 3.06 [a]
D	74.30 ± 9.45 [a]	86.98 ± 8.44 [a]	92.70 ± 9.53 [a]	98.21 ± 9.44 [a]
Control	58.67 ± 16.09 [b]	68.01 ± 9.21 [b]	77.71 ± 8.49 [b]	80.81 ± 9.11 [b]

Values presented with different superscripts within columns are significantly different ($p<0.05$) by Tukey test.

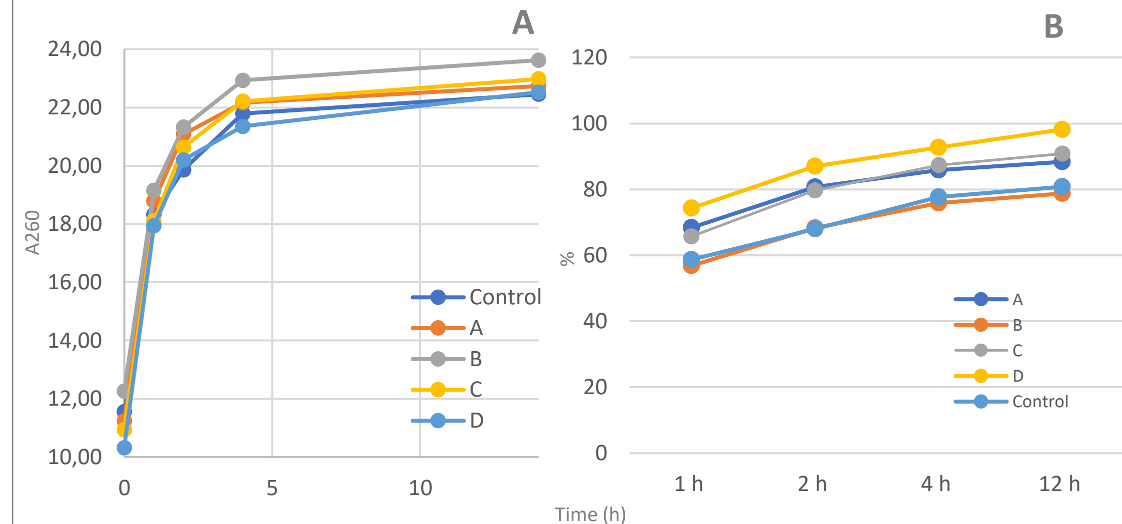

Figure 12 – Evolution of absorbance A260 in different PEFs treatments (A-D, control) (Before PEF, 1h after PEF, 2h, 4h and 12h).

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RESULTS

A280

- The A280 started (T0) at 10 (Fig.13 A).

- Extract D, 1h after PEF, obtained an absorbance 15% higher, compared with the control, which 12h after PEF increased to 17% (Fig.13 B).

- Despite extract D presented the highest value there were no significant differences (*p>0.05*) between the PEFs extracts A, C and D (Tab.6).

Table 6 – Evolution of A280 (%) in different PEFs treatments (A-D, control): 1h after PEF, 2h, 4h e 12h.

PEF	**1 h after PEF** (%) Mean ± SD	**2 h after PEF** (%) Mean ± SD	**4 h after PEF** (%) Mean ± SD	**12 h after PEF** (%) Mean ± SD
A	67.59 ± 14.39 [ab]	79.01 ± 14.21 [ab]	83.40 ± 15.21 [a]	85.58 ± 15.02 [a]
B	56.16 ± 9.83 [b]	66.58 ± 10.43 [b]	73.26 ± 10.60 [b]	76.23 ± 10.43 [b]
C	65.21 ± 0.82 [ab]	78.40 ± 2.81 [ab]	85.43 ± 2.34 [a]	88.81 ± 3.41 [a]
D	73.92 ± 9.49 [a]	85.91 ± 8.37 [a]	91.32 ± 9.74 [a]	96.64 ± 9.63 [a]
Control	58.80 ± 18.57 [ab]	67.43 ± 10.51 [b]	76.74 ± 9.74 [b]	79.40 ± 10.43 [b]

Values presented with different superscripts within columns are significantly different (p<0.05) by Tukey test.

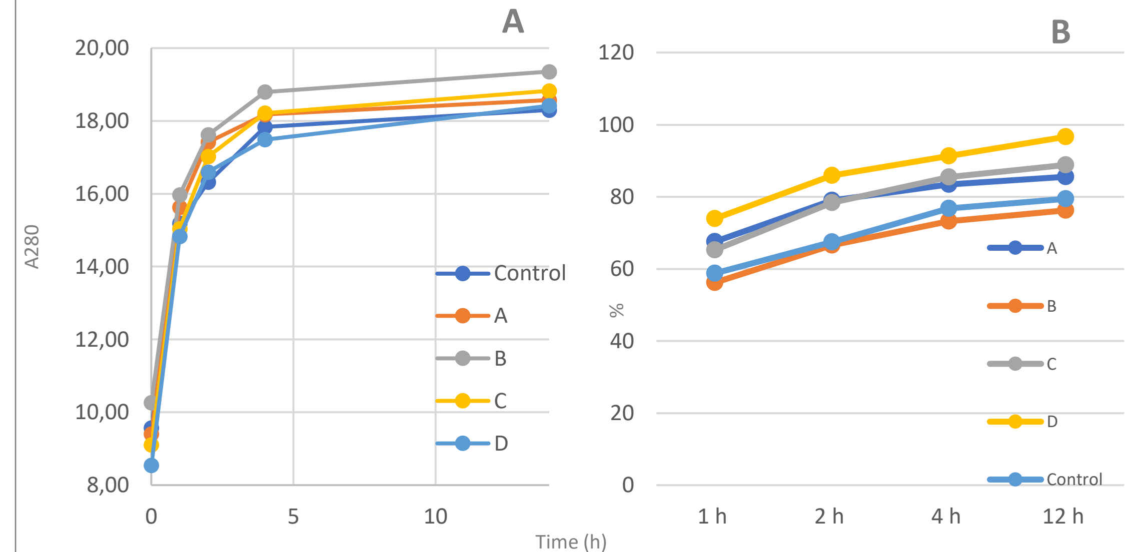

Figure 13 – Evolution of absorbance A280 in different PEF treatments (A-D, control) (Before PEF; 1h after PEF; 2h; 4h and 12h).

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RESULTS

A320

- The A320 started (T0) at 8 (Fig.14 A).
- Extract D, 1h after PEF, obtained an absorbance 15% higher, compared with the control, which 12h after PEF increased to 18% (Fig.14 B).
- Despite extract D presented the highest value there were no significant differences (*p>0.05*) between the PEFs extracts A, C and D (Tab.7).

Table 7 – Evolution of A320 (%) in different PEFs treatments (A-D, control): 1h after PEF, 2h, 4h e 12h.

PEF	**1 h after PEF** (%) Mean ± SD	**2 h after PEF** (%) Mean ± SD	**4 h after PEF** (%) Mean ± SD	**12 h after PEF** (%) Mean ± SD
A	67.77 ± 14.84 [ab]	76.31 ± 14.60 [ab]	78.13 ± 15.63 [a]	78.25 ± 15.56 [a]
B	54.64 ± 10.06 [b]	62.39 ± 10.55 [b]	66.56 ± 10.68 [b]	67.46 ± 10.47 [b]
C	64.21 ± 1.22 [ab]	74.66 ± 3.02 [ab]	79.27 ± 2.32 [a]	80.77 ± 3.61 [a]
D	74.44 ± 10.26 [a]	83.79 ± 8.78 [a]	86.55 ± 9.75 [a]	90.29 ± 9.99 [a]
Control	58.19 ± 19.90 [ab]	64.59 ± 11.28 [b]	71.81 ± 10.39 [b]	72.28 ± 11.32 [b]

Values presented with different superscripts within columns are significantly different ($p<0.05$) by Tukey test.

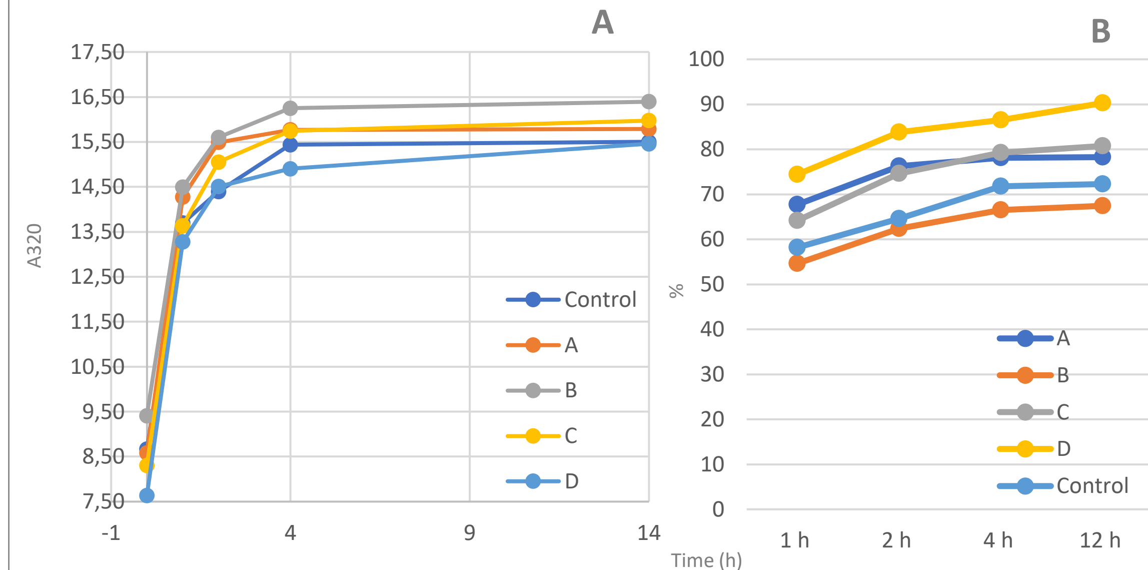

Figure 14 – Evolution of absorbance A320 in different PEFs treatments (A-D, control) (Before PEF; 1h after PEF; 2h; 4h and 12h).

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RESULTS

ELECTROPHORESIS

- Electrophoresis PAGE-Native permits distinguish protein profiles of cardosins (A, A0 and B) and concentrations obtained from PEFs extracts.
- PEFs extracts presented variation of cardosins concentrations between treatments and over time (Fig.15).
- The flowers of the PEFs batches, after 1h and 48h, were macerated and analyzed by electrophoresis to quantify the remaining proteins inside the flowers (Fig.16).

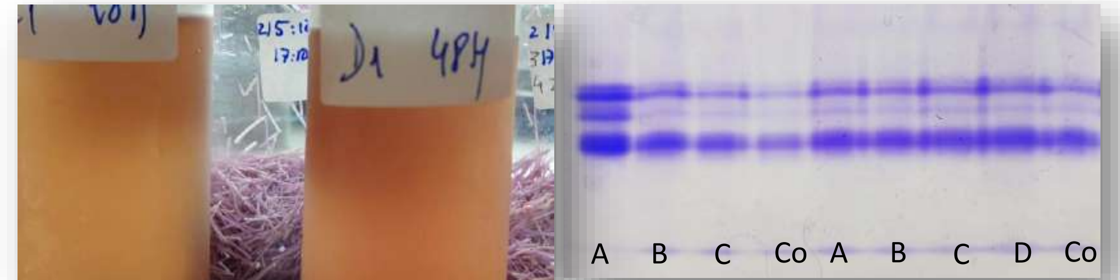

Figure 15 – Extracts from distinct PEFs treatments (A-D; control): 1h after PEF and 4h after PEF.

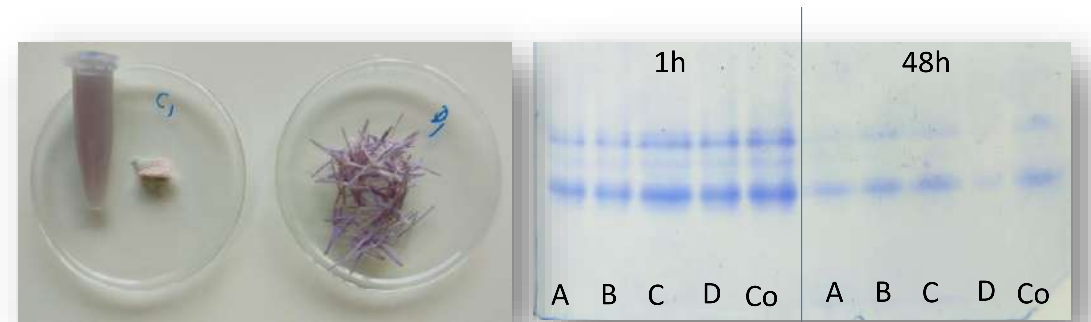

Figure 16 – Flower extracts after distinct PEFs treatments (A-D; control): 1h after PEF and 48h after PEF.

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RESULTS AND DISCUSSION

PROTEIN CONCENTRATION

- In curd, treatment D showed the highest protein concentration (dw) (39.2%) and B the lowest (35.8%) (Fig.17).
- In whey, treatments B, C and D presented the highest protein concentration (dw) (~36%) and A and control (~33%) the lowest (Tab.8; Fig.17).
- The intensity of the proteolytic activity in coagulation is directy related with the structure of the curd matrix.
- An intense proteolysis of milk caseins reduce the protein retention in the curd promoting a higher elution to the whey and a lower cheese yield.

Table 8 – Physicochemical analyses (dw) of curd and whey obtained from coagulation with distinct PEFs extracts (A-D) and control.

	Dry Matter Mean ± SD	Fat Mean ± SD	Protein Mean ± SD	Total Sugar Mean ± SD	Ash Mean ± SD	pH Mean ± SD
Milk	18.49 ± 0.05	46.58 ± 0.32	33.53 ± 0.22			
Curd A	30.88 ± 1.03	48.76 ± 2.26	38.68 ± 0.83	1.02 ± 0.39	8.44 ± 0.22	5.41 ± 0.23
Whey A	8.15 ± 0.03	17.32 ± 0.50	33.44 ± 0.02			
Curd B	30.95 ± 0.49	50.67 ± 0.99	35.81 ± 0.20	0.72 ± 0.18	6.96 ± 0.03	6.19 ± 0.05
Whey B	7.87 ± 0.11	10.51 ± 0.43	36.13 ± 0.15			
Curd C	30.25 ± 0.29	49.53 ± 0.56	38.80 ± 0.73	0.47 ± 0.24	8.17 ± 0.16	5.91 ± 0.13
Whey C	7.70 ± 0.13	7.91 ± 0.52	36.12 ± 0.48			
Curd D	29.85 ± 0.53	49.45 ± 0.66	39.16 ± 1.07	0.73 ± 0.28	8.28 ± 0.09	6.05 ± 0.02
Whey D	7.59 ± 0.18	7.78 ± 0.63	35.92 ± 0.59			
Curd Control	30.30 ± 0.59	48.69 ± 2.35	39.04 ± 1.58	1.88 ± 1.02	8.21 ± 0.04	6.11 ± 0.05
Whey Control	8.06 ± 0.03	15.51 ± 0.21	34.15 ± 0.41			

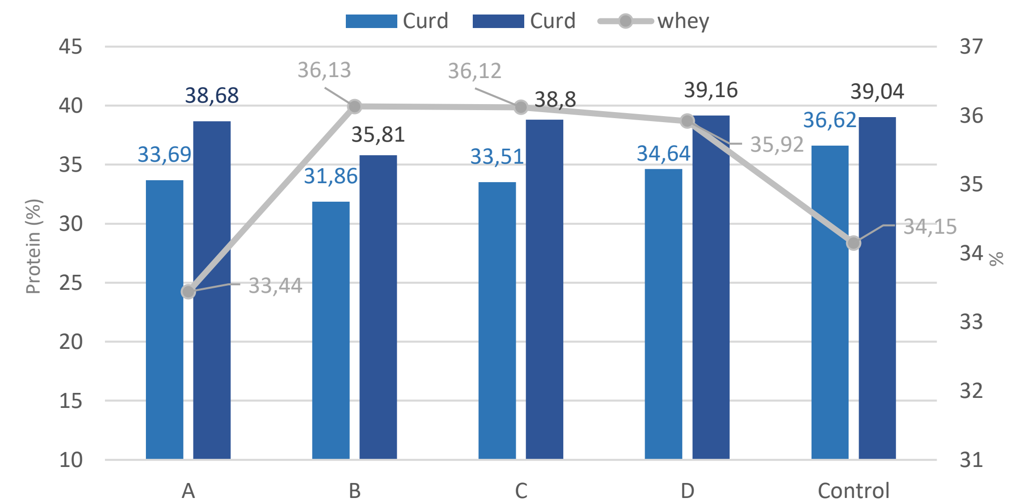

Figure 17 – Protein concentration (dw) of curd and whey from distinct PEFs extracts (A-D, control).

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RESULTS AND DISCUSSION

FAT CONCENTRATION

- In curd, treatment B presented the highest fat concentration (dw) (50.7%) and control the lowest (48.7%) (Fig.18).
- In whey, treatment A presented the highest fat concentration (dw) (17.3%), followed by control (15.5%) and C and D the lowest concentration (~8%) (Tab.9; Fig.18).
- The curd matrix is also determinant for the fat concentration retained in the curd and consequently the remanescent released to the whey.

Table 9 – Physicochemical analyses (dw) of curd and whey obtained from coagulation with distinct PEFs extracts (A-D) and control.

	Dry Matter Mean ± SD	Fat Mean ± SD	Protein Mean ± SD	Total Sugar Mean ± SD	Ash Mean ± SD	pH Mean ± SD
Milk	18.49 ± 0.05	46.58 ± 0.32	33.53 ± 0.22			
Curd A	30.88 ± 1.03	48.76 ± 2.26	38.68 ± 0.83	1.02 ± 0.39	8.44 ± 0.22	5.41 ± 0.23
Whey A	8.15 ± 0.03	17.32 ± 0.50	33.44 ± 0.02			
Curd B	30.95 ± 0.49	50.67 ± 0.99	35.81 ± 0.20	0.72 ± 0.18	6.96 ± 0.03	6.19 ± 0.05
Whey B	7.87 ± 0.11	10.51 ± 0.43	36.13 ± 0.15			
Curd C	30.25 ± 0.29	49.53 ± 0.56	38.80 ± 0.73	0.47 ± 0.24	8.17 ± 0.16	5.91 ± 0.13
Whey C	7.70 ± 0.13	7.91 ± 0.52	36.12 ± 0.48			
Curd D	29.85 ± 0.53	49.45 ± 0.66	39.16 ± 1.07	0.73 ± 0.28	8.28 ± 0.09	6.05 ± 0.02
Whey D	7.59 ± 0.18	7.78 ± 0.63	35.92 ± 0.59			
Curd Control	30.30 ± 0.59	48.69 ± 2.35	39.04 ± 1.58	1.88 ± 1.02	8.21 ± 0.04	6.11 ± 0.05
Whey Control	8.06 ± 0.03	15.51 ± 0.21	34.15 ± 0.41			

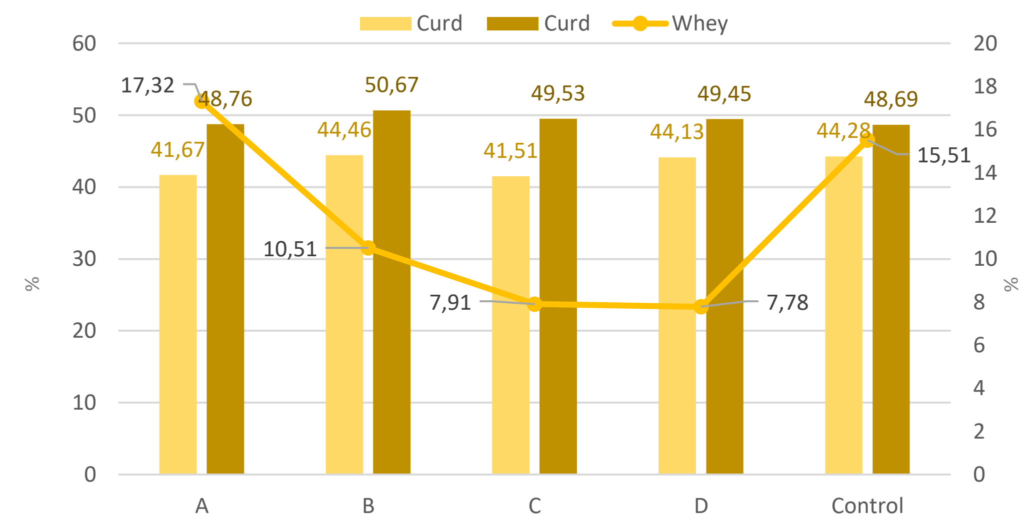

Figure 18 – Fat concentration (dw) of curd and whey from distinct PEFs extracts (A-D, control).

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RESULTS AND DISCUSSION

CURD - PROTEIN vs FAT

- In curd, treatment D presented the highest protein concentration (31%) and the lowest fat concentration (~46%) (Fig.19A).

- Treatments B and C, on the opposite, showed the highest fat content (~54%) and the lowest protein concentration (~27%).

- In curd, there seems to exist a relation between protein concentration and pH. Curd with high protein content presented a lower pH (Fig.19B).

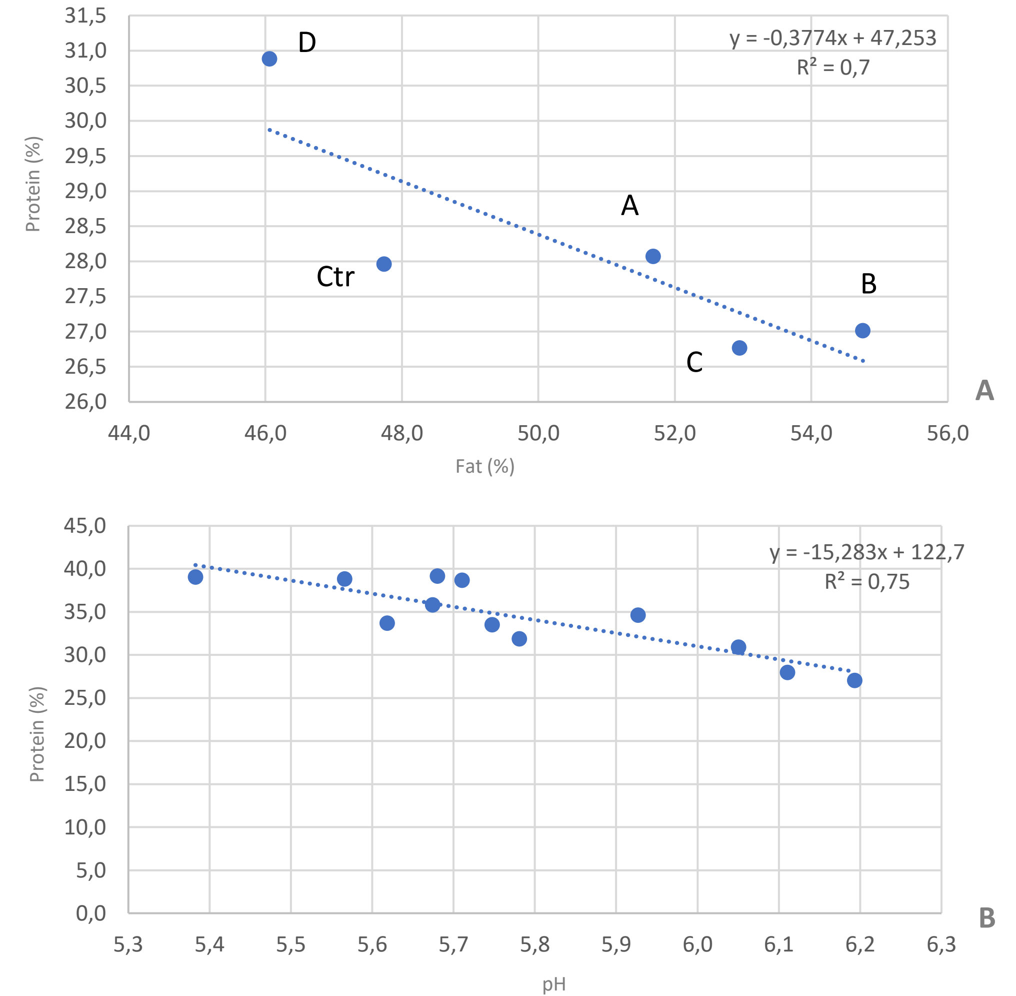

Figure 19 – (A) Fat and protein concentration (dw) of curd (A) with distinct PEFs extracts (A-D, control). (B) Relation between Protein and pH.

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RESULTS AND DISCUSSION

WHEY - PROTEIN vs FAT

- In whey, treatments A and B presented the highest protein concentration (dw) (~34%) and the lowest fat concentration (~17,5%) due the intense proteolytic activity in coagulation (Fig.20A).

- On the opposite, the control presented the lowest protein concentration (dw) (~28%) and the highest fat concentration (~23%).

- In whey, seems to exist a relation between fat (+) and protein (-) with dry matter contentes (Fig.20B).

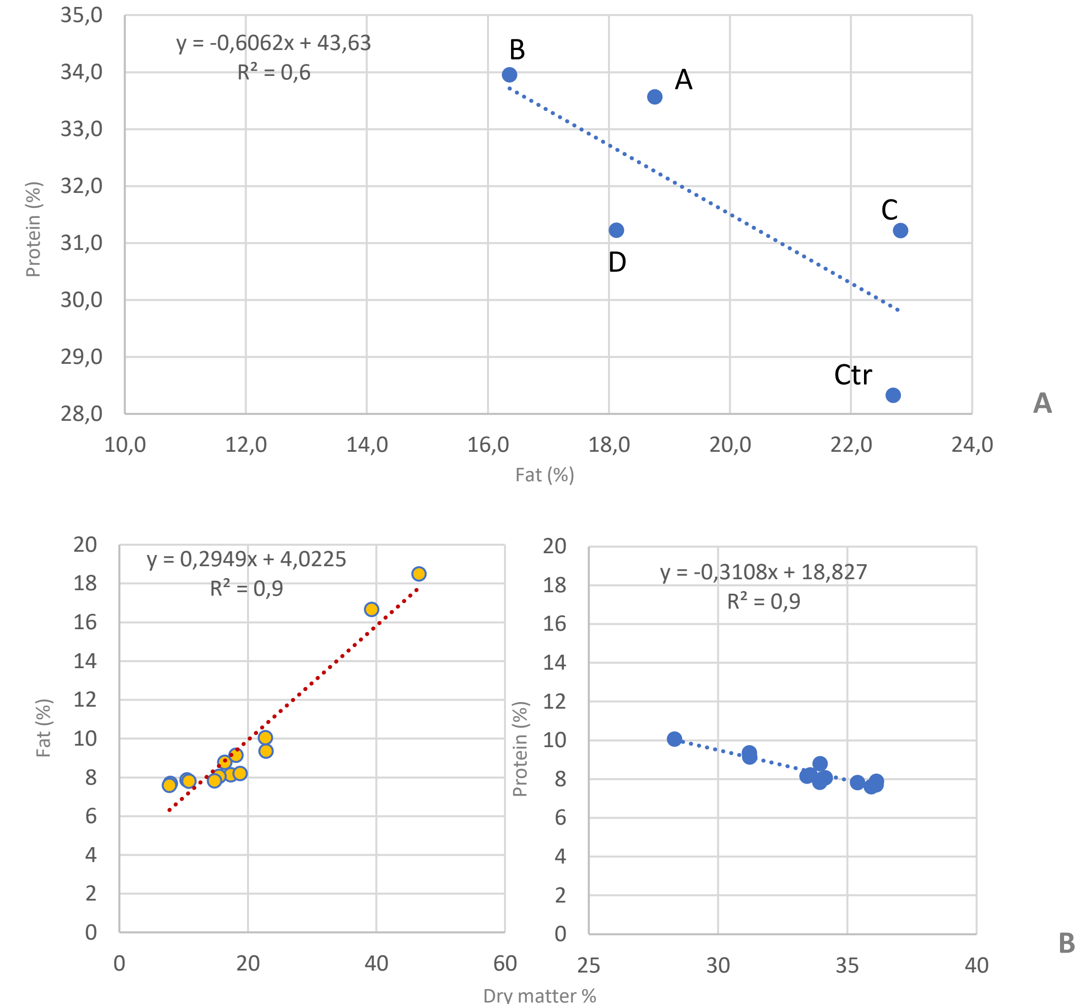

Figure 20 – (A) Fat and protein concentration (dw) of whey with distinct PEFs extracts (A-D, control). (B) Relation between fat and protein with dry matter.

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RESULTS AND DISCUSSION

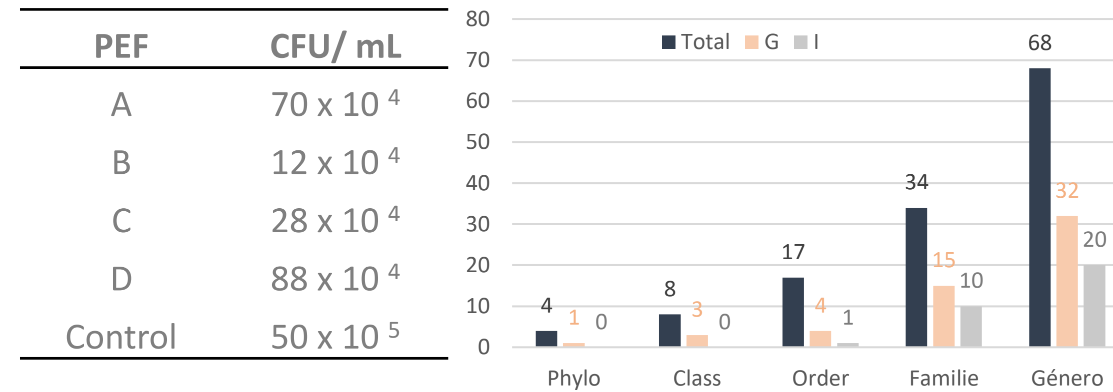
MICROBIOLOGICAL ANALYSIS

- 68 genera and 34 families of bacteria were identified in samples of dried cardoon flowers.

- PEFs treatments, presented a lower number of colonies forming units (CFU), significantly different *($p<0.05$)*, compared with the control (Tab.10).

- Treatments B and D presented the lowest and the highest values of microbial contamination, respectively.

Table 10 – Colonies forming unit (CFU/mL) in differents PEF treatments (A-D, control).



PEF	CFU/ mL
A	70×10^{4}
B	12×10^{4}
C	28×10^{4}
D	88×10^{4}
Control	50×10^{5}

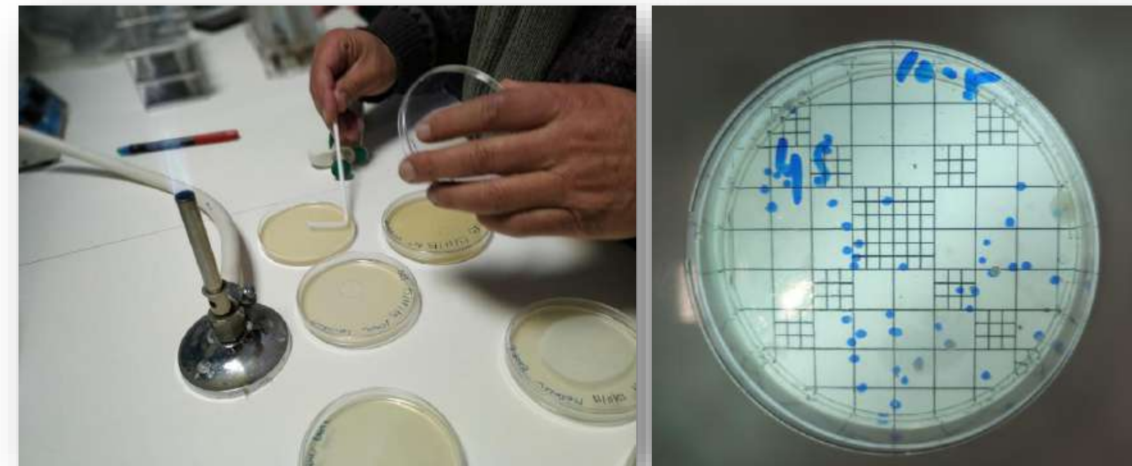


Figure 21 – Procedures of microbiological analysis.

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CONCLUSIONS

- The Pulse Electric Fields (PEFs), independently of the treatment (A-D) seems to increase the extraction of cardosins compared to the control.
- The highest differences between PEFs treatments and the control were obtained till 4h after PEF application (Fig.22).
- Treatment D presented the highest values in absorbance (A260, A280 and A320), however there were no significant differences between A, C and D.
- In curd, treatment D showed the highest protein concentration and the lowest fat concentration.

Figure 22 – Procedures of Pulse Electric Field analysis.

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FUTURE PROSPECTIVES

- The production of extracts of cardoon flowers from Serra da Estrela region with a standardized biochemical composition and microbiological safety is determinant to ensure the quality of coagulant extracts for PDO cheese production.

- PEFs extracts developed with technological and natural endogenous resources from Serra da Estrela PDO cheese region, enables a territorial marketing strategy.

- Further studies are still necessary to became the PEF assisted protein extraction from cardoon flowers as a competitive technology.

Figure 23 – Serra da Estrela Cheese PDO Region

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