coatings

Supplementary Materials

S.1. Determination of pH and Ash Content in Bee Pollen, Milk Samples, and Yoghurt Samples

S.1.1. Determination of pH

pH of bee pollen, milk, and yoghurt samples was measured in a 10% (*w*/*v*) aqueous solutions using a Delta OHM, model HD 3456.2, pH-meter (Padova, Italy) with a precision of 0.002 pH units. The instrument was calibrated with a buffer solution (pH = 7.0 ± 0.002, Cat. 22835-49) prior to measurements, which was obtained from HACH (Manchester, UK). Results reported are the average ± standard deviation values of three replicates.

S.1.2. Determination of Ash Content

For the determination of ash content in milk samples 10 g of milk were weighted in a crucible and a few drops of acetic acid were added for the sedimentation of milk proteins. Then the sample was steamed in a water bath (Memmert, Germany) of 105 °C until dryness. The crucible with dried milk was then placed on an electric hot plate with low heat. Heating was followed progressively until milk samples carbonize without smoke. The crucible was then transferred to a muffle furnace with a temperature of 550 ± 25 °C and was maintained for 4 h. Afterwards, it was cooled to 200 °C, and placed in a desiccator to cool for 30 min. If carbon particle is seen in the residue before weighing, a few drops of water or nitric acid should be added to the sample and the procedure described above should be repeated. The same procedure was followed for the determination of ash content in bee pollen samples using an amount of 3 g. Each analysis was carried out in triplicate and results were averaged providing the standard deviation values. Ash content was expressed as g/100 g using the formula:

$$\text{Ash} = \left(\frac{m_1 - m_2}{m_3 - m_2}\right) \times 100$$

- m_1 is the weight of the crucible with the ash with unit of g;
- m_2 is the weight of the empty crucible with unit of g; and
- m_3 is the weight of the crucible with the sample with unit of g.

S.2. Extraction of Phenolic Compounds

Approximately 5 g of dried pollen grains were placed in a glass vial containing 40 mL of ethanol. The vial with the prepared solution (mother solution A: 125,000 mg L^{-1}) was wrapped with aluminium foil, vortexed for 5 min and then every 1 h for 8 h. Finally, it was left in a dark place at room temperature for 24 h until exhaustive extraction The following day the mother solution was filtered using a filter paper and further dilutions were prepared using ethanol: 15,625 (1:8 *v*/*v*), 31,250 (1:4 *v*/*v*), and 62,500 (1:2 *v*/*v*) mg L^{-1}. These solutions were used in order to estimate the antioxidant activity development with respect to increasing concentration of pollen ethanolic extracts and to estimate the effective concentration of ethanolic extract that could cause the maximum inhibition of the free radical [15]. All ethanolic extracts were wrapped with parafilm and aluminum foil prior to use. Additionally, the mother solution A was kept at –18 °C and used for the determination of phenolic compounds and total phenolic content (TPC).

S.3. Analysis of Bee Pollen Phenolic Compounds using High Performance Liquid Chromatography Electro Spray Ionization Mass Spectrometry (HPLC/ESI-MS)

The chromatographic analysis was performed using an Agilent, model 1100 series HPLC system; Agilent, CA, USA). The wavelengths used were 254 ± 2 nm, 280 ± 2, nm, 330 ± 2 nm, 450 ± 2 nm, and 463 ± 2 nm. Gradient elution was used at a flow rate of 1 mL/min using water and acetonitrile (Merck) as the mobile phase. Gradient elution was used beginning with 10% of acetonitrile then increasing to

30% for 20 min, further increasing to 40% at 30 min, to 50% at 35 min, and finally to 50% at 40 min. The column was eluted isocratically for 10 min before next injection. Separation of the phenolic compounds was carried out using an Eclipse XDB C18 reversed phase column (Merck; 150 mm × 4.6 mm × 5 μm) at 25 °C.

The mass spectrometer was the LC/MSD trap SL (Agilent). The MS conditions were as follows: Injection volume: 3.5 μL; source conditions: drying gas (nitrogen) 8 L min^{-1} at 330° C; nebulizer pressure: 50 psi; mass range: 100–1000; scan mode: positive (+) and negative (-). Identification of phenolic compounds was achieved by comparing the mass to charge values [M-H^+] or [M-H^-] of individual peaks shown at total ion chromatograms with those identified previously in the literature. Analysis of bee pollen samples was run in triplicate (n = 3).

S.4. Determination of In Vitro Antioxidant Capacity of Bee Pollen Ethanolic Extracts and Pollen Enriched Yoghurts

The antioxidant capacity of pollen ethanolic extracts and pollen based yoghurts was estimated *in vitro* using the [DPPH•] assay according to the methodology described in previous studies [7,15] with modifications. The whole experimental procedure is given in Supplementary Material. More specifically, a volume of 2.8 mL of [DPPH•] solution (0.29 mM) plus 0.20 mL of the acetate buffer were placed in a cuvette (final volume of 3 mL) and the absorbance of the [DPPH•] radical was measured at t = 0 (A_0). Subsequently, 1.0 mL of each of the pollen ethanolic extracts (500, 1000, 1500, 2000, and 10,000 mg L^{-1}) were placed in the respective cuvettes plus 1.8 mL of the DPPH and 0.20 mL of the acetate buffer (final volume of the reaction medium equal to 3 mL). The absorbance was measured every 30 min until the value reached a plateau (steady state, A_t). The absorbance of the reaction mixture was measured at 517 nm.

The [DPPH•] antioxidant capacity with respect to each ethanolic extract concentration was calculated using the following equation:

$$\%AC = \left(\frac{A_0 - A_t}{A_0}\right) \times 100$$

where A_0 is the initial absorbance of the [DPPH•] free radical standard solution and A_t is the absorbance of remaining [DPPH•] free radical after reaction with pollen antioxidants, at steady state. The plateau of the reaction medium was reached early, at 1 h. Each analysis was run in triplicate. For this antioxidant test, ethanol plus buffer (2:1 v/v) was used as the blank.

For the antioxidant test of the control and prepared yoghurts enriched with pollen the respective reaction mixture was consisted of 1.8 mL of the [DPPH•], 1.0 mL of the acetate buffer and 0.20 mL of the conventional (control) and bee pollen based yoghurts (final volume of the reaction medium equal to 3 mL), respectively. All yoghurt samples were homogenized in a bag mixer using BagMixer apparatus [P (metal door/porte pleine), Serial No 00089381, Interscience, St Nom la Bretèche, France]. Prior absorbance measurements all prepared samples were filtered using Whatman filters (GD/X 25 mm Syringe Filter, Nylon 0.45 μm, w/GMF, G E Healthcare Whatman, Buckinghamshire, UK) with a pore size of 0.45 μm.

S.5. Results and Discussion

S.5.1. Chemical Composition and Physico-chemical Properties of Bee Pollen and Milk Types

pH of Yoghurt Samples

- 4.22 ± 0.02 (control), 4.27 ± 0.01 (supplemented with bee pollen 0.5% w/w), 4.27 ± 0.01 supplemented with bee pollen 1% w/w), 4.27 ± 0.01 (supplemented with bee pollen 2.5% w/w) and 4.27 ± 0.01 (supplemented with bee pollen 3% w/w) for cow yoghurts;
- 4.35 ± 0.02 (control), 4.39 ± 0.01 (supplemented with bee pollen 0.5% w/w), 4.39 ± 0.01 (supplemented with bee pollen 1% w/w), 4.39 ± 0.01 (supplemented with bee pollen 2.5% w/w) and 4.27 ± 0.01 (supplemented with bee pollen 3% w/w) for goat yoghurts;

- 4.01 ± 0.01 (control), 4.05 ± 0.01 (supplemented with bee pollen 0.5% *w*/*w*), 4.05 ± 0.02 (supplemented with bee pollen 1% *w*/*w*), 4.05 ± 0.01 (supplemented with bee pollen 2.5% *w*/*w*), and 4.05 ± 0.01 (supplemented with bee pollen 3% *w*/*w*) for sheep yoghurts. The measured pH values confirm the lactic acid fermentation during yoghurt preparation.

Table S1. Typical composition and physicochemical properties of bee pollen and milk types used for the preparation of functional yoghurts. Data involving total fat, saturated fat, protein, sugars, fibre, and salt contents were provided by the supplier.

Food Matrices	Fat (g/100 g)	Saturated Fat (g/100 g)	Protein (g/100 g)	Sugars (g/100 g)	Fibre (g/100 g)	Salt (g/100 g)	Ash (g/100 g)	pH
Bee pollen	7.00	2.30	17.60	61.00	8.40	0.03	2.28 ± 0.01	4.70 ± 0.01
Cow milk	3.80–4.20	2.60	3.50	4.90	nd	0.20	0.68 ± 0.01	6.62 ± 0.01
Goat milk	3.50	2.15	3.60	4.40	nd	0.08	0.81 ± 0.01	6.77 ± 0.01
Sheep milk	6.60	4.60	5.70	5.40	nd	0.04	0.95 ± 0.01	6.57 ± 0.04

Table S2. Significant parameters ($p < 0.05$) used for the development of the discrimination model as assessed by MANOVA.

Tests of Equality of Group Means					
Variables	**Wilks' Lambda**	**F**	**df1**	**df2**	***P***
Total phenolic content of conventional yoghurts (control samples)	0.000	494,107.133	2	19	0.000
Total phenolic content of yoghurts enriched with 0.5% (*w*/*v*) bee pollen	0.000	74,090.195	2	19	0.000
Total phenolic content of yoghurts enriched with 1.0% (*w*/*v*) bee pollen	0.000	170,809.528	2	19	0.000
Total phenolic content of yoghurts enriched with 2.50% (*w*/*v*) bee pollen	0.000	87,676.474	2	19	0.000
Total phenolic content of yoghurts enriched with 3.0% (*w*/*v*) bee pollen	0.000	1,029,057.724	2	19	0.000
In Vitro Antioxidant capacity of control samples	0.000	110,728.977	2	19	0.000
In Vitro Antioxidant capacity of yoghurts enriched with 0.5% (*w*/*v*) bee pollen	0.000	72,578.735	2	19	0.000
In Vitro Antioxidant capacity of yoghurts enriched with 1.0% (*w*/*v*) bee pollen	0.000	30,487.649	2	19	0.000
In Vitro Antioxidant capacity of yoghurts enriched with 2.50% (*w*/*v*) bee pollen	0.000	42,385.052	2	19	0.000
In Vitro Antioxidant capacity of yoghurts enriched with 3.00% (*w*/*v*) bee pollen	0.000	176,156.306	2	19	0.000
Taste scores of conventional yoghurts (control samples)	0.920	0.823	2	19	0.454
Taste scores of yoghurts enriched with 0.5% (*w*/*v*) bee pollen	0.478	10.364	2	19	0.001
Taste scores of yoghurts enriched with 1.0% (*w*/*v*) bee pollen	0.468	10.787	2	19	0.001
Taste scores of yoghurts enriched with 2.50% (*w*/*v*) bee pollen	0.981	0.185	2	19	0.832
Taste scores of yoghurts enriched with 3.00% (*w*/*v*) bee pollen	0.975	0.241	2	19	0.788
Odour scores of conventional yoghurts (control samples)	0.558	7.537	2	19	0.004
Odour scores of yoghurts enriched with 0.5% (*w*/*v*) bee pollen	0.527	8.517	2	19	0.002

Table S2. *Cont.*

Tests of Equality of Group Means					
Variables	**Wilks' Lambda**	**F**	df_1	df_2	***P***
Odour scores of yoghurts enriched with 1.0% (*w*/*v*) bee pollen	0.190	40.455	2	19	0.000
Odour scores of yoghurts enriched with 2.50% (*w*/*v*) bee pollen	0.829	1.955	2	19	0.169
Odour scores of yoghurts enriched with 3.00% (*w*/*v*) bee pollen	0.524	8.636	2	19	0.002
Appearance scores of conventional yoghurts (control samples)	0.370	16.193	2	19	0.000
Appearance scores of yoghurts enriched with 0.5% (*w*/*v*) bee pollen	0.389	14.924	2	19	0.000
Appearance scores of yoghurts enriched with 1.0% (*w*/*v*) bee pollen	0.973	0.264	2	19	0.771
Appearance scores of yoghurts enriched with 2.50% (*w*/*v*) bee pollen	0.980	0.195	2	19	0.825
Appearance scores of yoghurts enriched with 3.0% (*w*/*v*) bee pollen	0.723	3.631	2	19	0.046
Cohesion scores of conventional yoghurts (control samples)	0.412	13.536	2	19	0.000
Cohesion scores of yoghurts enriched with 1.0% (*w*/*v*) bee pollen	0.478	10.364	2	19	0.001
Cohesion scores of yoghurts enriched with 2.50% (*w*/*v*) bee pollen	0.638	5.381	2	19	0.014
Cohesion scores of yoghurts enriched with 3.00% (*w*/*v*) bee pollen	0.606	6.172	2	19	0.009

df: degrees of freedom; F: Fisher's coefficient; P: probability.

S.5.2. Estimation of the Parameters that Built the Regression Analysis Modelling Equations

R-squared can be defined as follows:

$$R^2 = 1 - SS_{resid}/SS_{model} + SS_{resid} = 1 + SS_{error}/SS_{total} \quad (1)$$

The R_{pred}^2 and the R_{adj}^2 have been obtained using Equations (2) and (3):

$$R^2_{adj} = 1 - (n - 1/n - p)\,(SS_{error}/SS_{total}) = 1 - (n-1)/(n-p)(1-R^2) \quad (2)$$

$$R^2_{pred} = (1 - PESS)/(SS_{total} - SS_{block}) \quad (3)$$

where n is the number of experiments, p is the number of model parameters including intercept and any block coefficient, and PESS is the prediction error of sum of squares:

$$PESS = \sum_{i=1}^{n}(e_{i,-i})2,\ e_{i,-i} = y_i - \bar{y}_{i,-i} \quad (4)$$

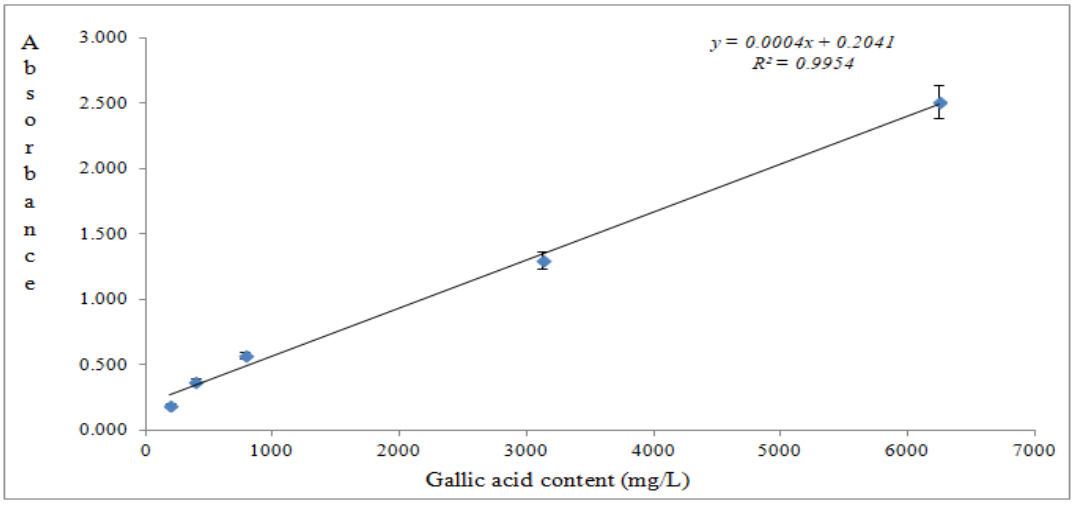
where $e_{i,-i}$ is residual, y_i is the experimental value, and $\bar{y}_{i,-i}$ is the predicted value.

The adjusted-R^2 is a comparative measure of the suitability of alternative nested sets of measured variables. Adjusted-R^2 is particularly useful in the feature selection stage of model building. A higher adjusted-R^2 indicates that the most significant parameters are used in the model building.

Table S3. Discriminatory power of the statistical model developed for the classification of conventional and functional yoghurts based on specific bee pollen amounts.

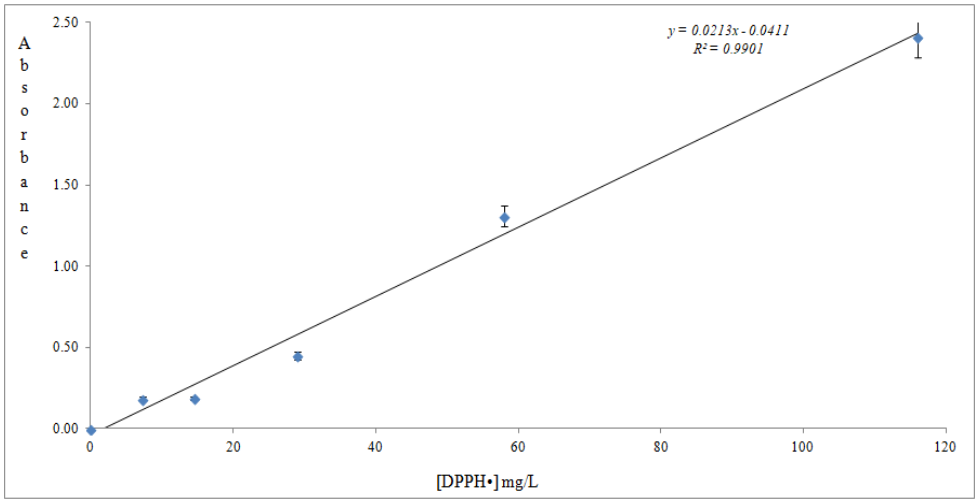
Classification Results [a,b,c]						
Chemometric Technique		**Milk Type**	**Predicted Group Membership**			**Total**
			Yoghurts Prepared from Cow Milk	**Yoghurts Prepared from Goat Milk**	**Yoghurts Prepared from Sheep Milk**	
Original[a]	Count	Yoghurts prepared from cow milk	10	0	0	10
		Yoghurts prepared from goat milk	0	6	0	6
		Yoghurts prepared from sheep milk	0	0	6	6
		Ungrouped cases	0	4	4	8
	%	Yoghurts prepared from cow milk	100.0	.0	.0	100.0
		Yoghurts prepared from goat milk	.0	100.0	.0	100.0
		Yoghurts prepared from sheep milk	.0	.0	100.0	100.0
		Ungrouped cases	.0	50.0	50.0	100.0
Cross-validated[b]	Count	Yoghurts prepared from cow milk	10	0	0	10
		Yoghurts prepared from goat milk	0	6	0	6
		Yoghurts prepared from sheep milk	0	0	6	6
	%	Yoghurts prepared from cow milk	100.0	.0	.0	100.0
		Yoghurts prepared from goat milk	.0	100.0	.0	100.0
		Yoghurts prepared from sheep milk	.0	.0	100.0	100.0

[a]100.0% of original grouped cases correctly classified; [b] Cross-validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case; [c] 100.0% of cross-validated grouped cases correctly classified.

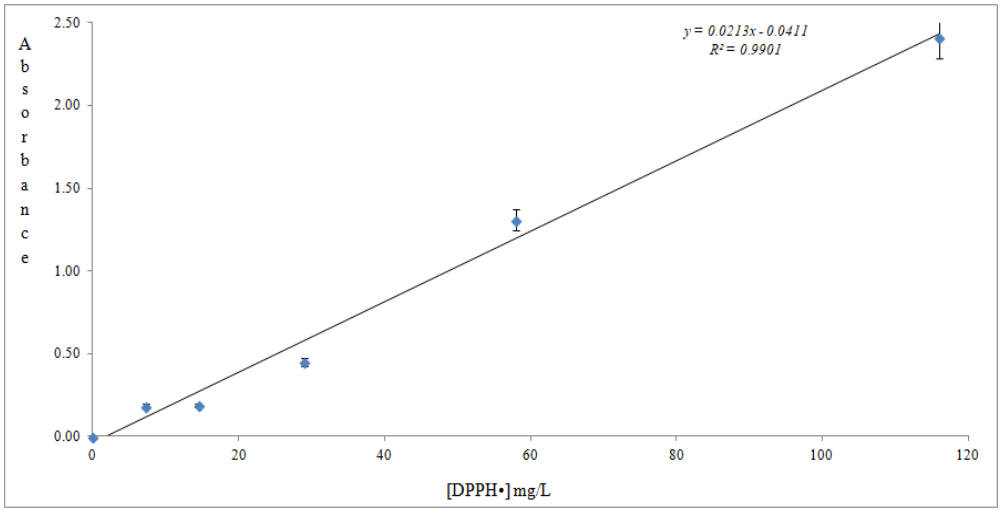


(**a**)

Figure S1. *Cont.*



(**b**)



(**c**)

Figure S1. (**a**) Calibration curve of gallic acid. *X*-axis: concentration of gallic acid solutions (mg L^{-1}). *Y*-axis: absorbance. Error bars are provided at the confidence level of $p < 0.05$; (**b**) Calibration curve of [DPPH•] radical ethanolic solution. *X*-axis: concentration of free radical (mg L^{-1}). *Y*-axis: absorbance. Error bars are provided at the confidence level of $p < 0.05$; (**c**) Development of total phenolic content (mgGAE L^{-1}) of Greek bee pollen with respect to ethanolic extracts (mg L^{-1}). Error bars are provided at the confidence level $p < 0.05$.