Monitoring Commercial Starter Culture Development in Presence of Red Grape Pomace Powder to Produce Polyphenol-Enriched Fresh Ovine Cheeses at Industrial Scale Level

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Abstract: Red grape Nero d'Avola cultivar grape pomace powder (GPP) was applied during fresh ovine cheese production in order to increase polyphenol content. Before cheeses were produced, the bacteria of a freeze-dried commercial starter culture were isolated and tested in vitro against GPP. Two dominant strains, both resistant to GPP, were identified. The starter culture was inoculated in pasteurized ewe's milk and the curd was divided into two bulks, one added with 1% (w/w) GPP and another one GPP-free. GPP did not influence the starter culture development, since lactic acid bacteria (LAB) counts were 10^9 CFU/g in both cheeses at 30 d. To exclude the interference of indigenous LAB, the pasteurized milk was analyzed, and several colonies of presumptive LAB were isolated, purified and typed. Four strains were allotted into *Enterococcus* and *Lacticaseibacillus* genera. The direct comparison of the polymorphic profiles of cheese bacteria evidenced the dominance of the starter culture over milk LAB. The addition of GPP increased cheese total phenolic compounds by 0.42 g GAE/kg. Sensory evaluation indicated that GPP-enriched cheese was well appreciated by the judges, providing evidence that GPP is a suitable substrate to increase the availability of total phenolic content in fresh ovine cheese.

Keywords: grape pomace powder; commercial starter culture; ovine cheese; industrial application; total phenolic content

1. Introduction

Polyphenols are secondary plant-derived bioactive metabolites [1]. These compounds are able to prevent lipid oxidation, deterioration of polyunsaturated fatty acids and inhibit the growth of undesired pathogenic and spoilage microorganisms in food products [2,3]. Polyphenols also exert beneficial effects on the human health, including the prevention of different chronic diseases [4]. In the last decades, in light of the directives from the European Commission (1999/31/EC and 2008/98/EC) related to waste and environmental sustainability, fruit and vegetable processing industries have focused their efforts to diminish the environmental impact of wastes and by-products. One promising strategy to valorize these organic matrices is the extraction of active compounds [5,6]. In this context, particular attention has been paid to vinery by-products [7], in particular grape pomace, which represents approximately 20–25% of the grapes at harvest [8]. Grape pomace contain grape skin and seeds [9] and represents a waste biomass difficult to dispose of [10], since it cannot be directly applied as a soil conditioner or fertilizer due to high levels of phenolic compounds inhibiting the germination of plant seeds [11] and the growth of soil microor-

ganisms [12]. Grape pomace cannot be used in animal feed either because the presence of lignin limits its digestibility [13]. However, due to the increasing consumer request of functional foods [14], grape pomace in powder form, namely grape pomace powder (GPP), has been added to foods containing low levels of polyphenols and dietary fiber in order to improve their functional and nutraceutical properties [15].

Cheese is consumed almost worldwide and represents an important source of proteins, minerals, vitamins and fatty acids [16], but it is poor in bioactive compounds [17]. The enrichment of cheese with polyphenols has been recognized as a good strategy to produce healthy and functional cheeses [18].

To this purpose, fruit and vegetables by-products such as artichoke external leaves, broccoli stems and leaves, corn bran, tomato peel [19] and wheat bran [20] have been powdered and used in cheese production. The use of GPP for making dairy products is not new. The addition of GPP in bovine dairy products such as yogurt [21,22] and semihard and hard cheeses [23] has been investigated and their effect on the nutritional quality of the processed foods evaluated. Very recently, the addition of GPP to fresh ewe's milk cheese has been investigated using single *Lactococcus lactis* strains at the pilot plant scale level [24], but no industrial production has been carried out. In order to perform high volume cheese production, commercial starter cultures are of paramount importance because they include several mixed-strain starters to overcome phage related issues [25].

This study is part of a project mainly aimed at finding commercial alternatives for the valorization of ovine cheeses as well as reuse of winemaking industry by-products. With this in mind, the specific objectives of the present work were to: (i) evaluate the in vitro inhibitory activity of GPP against commercial starter culture; (ii) monitor the persistence of GPP during the manufacture of GPP-enriched fresh ovine cheese; (iii) evaluate the total polyphenols content of final cheeses; (iv) evaluate the sensory characteristics of the final cheeses.

2. Materials and Methods

2.1. Raw Material and Starter Culture

GPP was prepared from grape pomace of *Vitis vinfera* L cv. Nero d'Avola, provided by the agricultural cooperative "Cantine Europa" located in Petrosino (Trapani, Italy) at the end of the 2020 vintage, as reported by Marchiani et al. [23]. Briefly, grape pomace was dried in a semi-industrial oven Compact Combi (Electrolux, Pordenone, Italy) at 54 °C for 48 h and milled through a Retsch apparatus (Haan, Germany) to a particle size of 250 μm. The whole ewe's milk used for cheese production was obtained from the artisanal dairy farm Biopek, located in Gibellina (Trapani, Italy), with sheep of Valle del Belìce breed. Commercial freeze-dried starter preparation (LYOBAC-D NT, Alce International s.r.l., Quistello, Italy) was composed of two defined strains of *Lactococcus lactis* as indicated by the producer company.

2.2. Isolation and Typing of Commercial Starter Culture

Freeze-dried starter preparation (5 g) was first homogenized with Ringer's solution (45 mL) by a stomacher Bag-Mixer 400 (Interscience, Saint Nom, France) for 2 min at the maximum speed and then serially diluted (1:10). Cell suspensions of freeze-dried starter culture were subjected to a plate count for the enumeration of mesophilic coccus-shaped LAB on M17 agar (Biotec, Grosseto, Italy), incubated anaerobically for 48 h at 30 °C. After growth, the colonies of presumptive lactic acid bacteria (LAB) were randomly picked up, purified by successive subculturing and tested for the Gram reaction [26], and for catalase activity performed by addition of H_2O_2 (3% (*v/v*)) to the colonies [27]. The purity and cell morphology of the isolates were microscopically investigated. All cultures were subjected to DNA extraction using a DNA-SORB-B kit (Sacace Biotechnologies Srl, Como, Italy) following the protocol provided by the manufacturer. The differentiation of the isolates at the strain level was performed by randomly amplified polymorphic DNA (RAPD)-PCR analysis using single primers M13 (5′-GAGGGTGGCGGTTCT-3′), AB106 (5′-

TGCTCTGCCC-3′) and AB111 (5′-GTAGACCCGT-3′). Polymerase chain reactions (PCRs) were performed applying the protocol described by Gaglio et al. [28].

2.3. Inhibitory Assay

In order to test the suitability of GPP to produce fresh ovine cheese, its potential inhibitory activity was tested against the strains isolated from the commercial starter culture. GPP was rehydrated in distilled water to a final concentration of 200 µg/mL as commonly performed for extracts of vegetable origin [29,30]. The water-soluble extract of GPP was tested against a cell density of 10^7 CFU/mL of each LAB strain in M17 soft agar (0.7% *w/v*) applying the paper disc diffusion assay as reported by Cruciata et al. [31]. Streptomycin (10% *w/v*) was used as positive control, with sterile water as negative control. The inhibitory activity was considered positive if a definite clear area was detected around the paper discs. This test was performed in duplicate.

2.4. Cheese Productions and Sample Collection

Cheese production was performed under industrial conditions at a dairy factory scale (Biopek, Gibellina, Italy) applying "Primosale" type pressed cheese technology. The experimental plan included two different cheese productions: a control production (CP) inoculated with the same commercial starter culture characterized for GPP resistance, and an experimental production (EP) inoculated with the commercial starter culture and 1% (*w/w* of curd) of GPP. Each trial was performed in a stainless-steel vat previously sanitized with a PROMOX P900 solution (Leggiuno, Italy), with 500 L of pasteurized ewe's milk (72 °C for 15 s). Freeze-dried commercial starter culture (5 U) was reactivated in 5 L of pasteurized ewe's milk and kept for 10 min at 44 °C. After cooling at 38 °C, the bulk milk was inoculated with the starter culture preparation and kept under slow agitation for 30 min prior to adding liquid rennet (150 mL, Fromase® 220 TL, DSM Bright Science Brighter Living, Heerlen, The Netherlands). After 40 min, the coagulum was mechanically cut to the dimension of rice grains. After draining whey, the control curd was immediately put into perforated plastic molds, while GPP was added to the experimental curd and then transferred into perforated plastic molds. Both curds were stewed at 40 °C for 2 h and turned every 30 min. At the end of the process, all cheeses were salted in saturated brine for 6 h, dried for 24 h at room temperature and ripened for 30 d at 13 °C and 80% relative humidity. The trials were carried out in triplicate over three consecutive weeks. Samples of GPP, raw milk, pasteurized milk, inoculated milk after addition of reactivated starter culture, curds, and cheeses after 30 d of ripening, were collected for analyses.

2.5. Microbiological Analyses

All milk samples (1 mL) were serially diluted directly in Ringer's solution (Sigma-Aldrich, Milan, Italy), while GPP, curd and cheese samples (10 g) were first homogenized in 90 mL of Ringer's solution in a stomacher and then serially diluted as reported above.

Cell suspensions of raw milk, pasteurized milk and GPP were subjected to plate counts for the enumeration of the following microbial groups: total mesophilic microorganisms (TMM) on plate count agar (PCA), incubated aerobically at 30 °C for 72 h; mesophilic rod shaped LAB on de Man–Rogosa–Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 mol/L) and incubated anaerobically for 48 h at 30 °C; mesophilic coccus-shaped LAB on Media 17 (M17) agar, incubated anaerobically for 48 h at 30 °C; members of the *Enterobacteriaceae* family on violet red bile glucose agar (VRBGA), incubated aerobically at 37 °C for 24 h; coagulase-positive staphylococci (CPS) on Baird-Parker (BP) agar added with rabbit plasma fibrinogen, incubated aerobically at 37 °C for 48 h; *Listeria monocytogenes* on *Listeria* selective agar base (LSAB) added with SR0140E supplement, incubated aerobically at 37 °C for 48 h; *Escherichia coli* and *Salmonella* spp., on Hektoen enteric agar (HEA), incubated aerobically at 37 °C for 24 h.

Cell suspensions of inoculated milk after addition of reactivated starter culture, curds and cheeses were analyzed for TMM and LAB exclusively on M17 agar as reported above.

All media and supplements were purchased from Biotec, expect HEA provided by Microbiol Diagnostici (Uta, Italy). Plates counts were performed in duplicate.

2.6. Isolation, Genotypic Differentiation and Identification of Thermoduric Indigenous Milk LAB

The colonies of presumptive LAB developed from the highest dilutions of pasteurized milk sample suspensions were picked up, purified and tested for main LAB characteristics as reported above. All Gram positive and catalase-negative cultures were considered presumptive LAB and differentiated by RAPD-PCR analysis as reported above. Strain typing was performed using the software Gelcompare II version 6.5 (Applied-Maths, Sin Marten Latem, Belgium). Genotypic identification was performed by sequencing the 16S rRNA gene following the procedures applied by Gaglio et al. [32] using the primers rD1 (5′-AAGGAGGTGATCCAGCC-3′) and fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′). The identities of the sequences were determined by comparison with those available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) [33] and EzTaxon-e (http://eztaxon-e.ezbiocloud.net/) [34] databases. The unequivocal identification of the *Enterococcus* species that showed a discrepancy between the two databases was confirmed using the multiplex PCR assay based on the *sodA* gene. PCR reactions were performed as described by Jackson et al. [35] using the primers FM1 (5′-GAAAAAACAATAGAAGAATTAT-3′) and FM2 (5′-TGCTTTTTTGAATTCTTCTTTA-3′) specific for *Enterococcus faecium*.

2.7. Commercial Starter Culture Recognition

The persistence of the commercial starter culture, and its dominance over the indigenous milk LAB resistant to the pasteurization process, was assessed by microscopic inspection and RAPD profile comparison between the LAB collected during cheese making and those purified from the commercial starter culture.

2.8. Total Phenolic Content

Total phenolic content (TPC) of GPP and cheese samples was evaluated through an analyzer iCubio iMagic M9 (Shenzhen iCubio Biomedical Technology Co. Ltd. Shenzhen, China), which runs with full automation. This system automatically pipetted reagents and samples into the cuvette, allowed incubation at a controlled temperature, read absorbance at the specific wavelength and calculated the concentration of the analyses with a calibration curve. The parameters used in the automated photometric systems were temperature, 37 °C; wavelengths 340 nm and 415 nm (bichromatic) and optical path of 1 cm. The reagent used was Enzytec™ Polyphenols Cod. E2530. All reagents and standards were purchased from R-Biopharm AG (Darmstadt, Germany). All samples were homogenized before the extraction procedure. Conventional solid-liquid extraction of polyphenols and sample preparation were performed according to manufacturer's instructions: 20 g of each sample were treated with 80 mL of ethanol solution (70% *v/v*) and stirred for 60 min at 25 °C. The samples were then filtered and put into a 100 mL volumetric flask, brought to volume with distilled water. The final concentration of polyphenols was then reported as g of gallic acid equivalent (GAE)/kg of dry mass (DM) for GPP or g GAE/kg of product for cheeses. All measurements were performed in duplicate.

2.9. Sensory Evaluation

The control and experimental cheese productions, at 30 d of ripening, were evaluated for their sensory traits by a descriptive panel of 12 judges (six women and six men aged between 21 and 68 years old) selected for their cheese consumption habits. The judges were trained for cheese attribute evaluation following the ISO 8589 [36] indications. For each cheese, the judges evaluated twelve descriptive attributes among those reported by Niro et al. [37] and evaluated by Costa et al. [38]. In particular, the evaluation considered the following aspects of the cheeses: intensity of odor and aroma, sweet, salt, acid, astringent, friability, fiber, adhesiveness, hardness, humidity and overall assessment. The quality of

each attribute was scored using a 70-mm visual analogue scale anchored on the left as reported by Faccia et al. [39].

2.10. Statistical Analyses

Microbiological data were subjected to One-Way Variance Analysis (ANOVA) using XLStat software version 7.5.2 for Excel (Addinsoft, New York, NY, USA). The Duncan test was applied to evaluate the level of significance between control and experimental samples. $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. Isolation and Typing of Commercial Starter Culture

Dried starter culture was dominated by LAB with levels of mesophilic LAB cocci of 10.5 Log CFU/g. LAB (Gram positive and catalase negative) were isolated from M17 agar media incubated at 30 °C at the highest dilutions of cell suspensions. After microscopic inspection, all isolates showed coccus-shaped cells organized in short chains, typical of lactococci [40]. As declared by the producer company, RAPD analysis confirmed the presence of two strains (Figure 1) included in the *L. lactis* freeze-dried starter preparation.
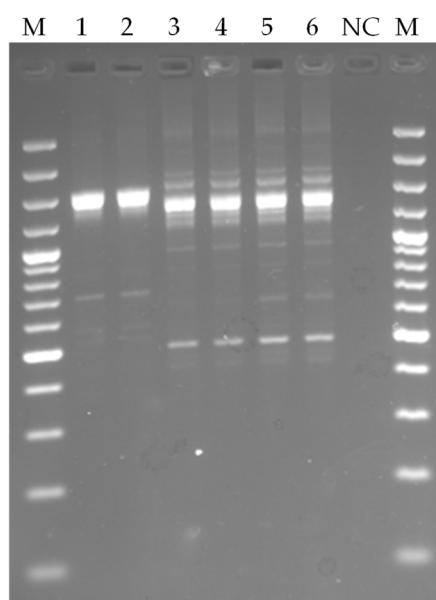

Figure 1. Image of agarose gel showing randomly amplified polymorphic DNA profiles of commercial starter lactic acid bacteria (LAB). Lanes M, GeneRuler 100 bp plus DNA ladder (Thermo Fisher Scientific, Inc., Vilnius, Lithuania); lanes 1-2, strain 1; lanes 3-6, strain 2; lane NC, negative control.

3.2. Evaluation of Suitability of GPP for Cheese Making

The suitability of GPP for the production of cheeses depends on its harmlessness towards LAB, which are the main fermentation agents necessary to transform milk into cheese [41]. The results of the paper disc diffusion assay showed that GPP did not inhibit the growth of the *L. lactis* strains isolated from the dried starter culture (results not shown), confirming its suitability in fresh ovine cheese production.

3.3. Microbiological Evolution during Cheese Productions

Microbiological investigation was carried out throughout cheese production from GPP to final 30-d ripened cheeses. No colonies of bacteria and yeasts were detected in GPP. The absence of these microorganisms is a consequence of the thermal treatment applied during the oven-drying process [42]. Statistically significant differences ($p < 0.001$) were observed for the levels of all microbial groups investigated between the raw milk and pasteurized

milk (Figure 2). The results of *L. monocygenes* and *Salmonella* spp. are not reported in the figure because their levels were below the detection limit for both matrices.
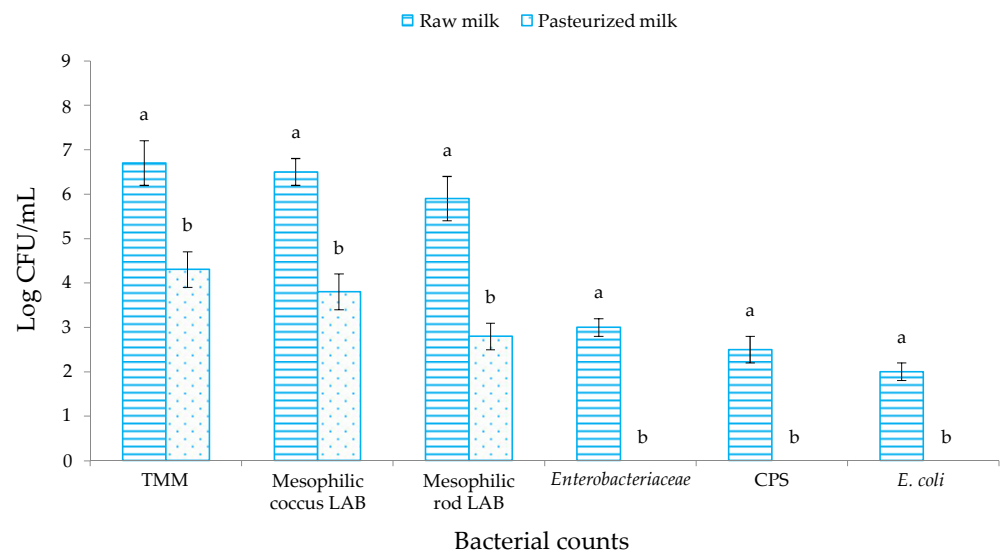

Figure 2. Microbial loads (Log CFU/mL) of raw and pasteurized milk samples. Abbreviations: TMM, total mesophilic microorganisms; CPS, coagulase-positive staphylococci; *E.*, *Escherichia*. Results indicate mean values and standard deviation of six determinations (carried out in duplicate for three independent productions). Different superscript letters indicate significant differences on microbial concentrations according to Duncan's test between raw and pasteurized milk samples for $p < 0.05$.

The levels of TMM, mesophilic coccus and rod LAB of raw milk were 6.7, 6.5 and 5.9 Log CFU/mL, respectively, while they were recorded at about 3 Log cycles lower in pasteurized milk. These results are not surprising since indigenous milk LAB are able to survive the heat treatment [43,44]. Regarding the undesired bacterial groups, especially members of *Enterobacteriaceae* family, CPS and *E. coli*, they were in the range 10^2–10^3 CFU/mL in raw milk and completely disappeared after pasteurization.

The growth of the fermenting agent, added as starter culture, during cheese productions is reported in Table 1.

Table 1. Growth of commercial starter LAB during cheese production.

Samples	Bacterial Counts	
	TMM	*L. lactis*
Inoculated milk	6.7 ± 0.4	6.8 ± 0.5
Curd		
CP	7.9 ± 0.2 [a]	7.8 ± 0.3 [a]
EP	7.7 ± 0.2 [a]	8.0 ± 0.2 [a]
p value	0.288	0.391
Cheese		
CP	9.3 ± 0.5 [a]	9.2 ± 0.2 [a]
EP	9.1 ± 0.2 [a]	9.1 ± 0.4 [a]
p value	0.555	0.718

Units are Log CFU/mL for liquid samples and Log CFU/g for solid samples. [a] Results indicate mean values ± S.D. of six plate counts (carried out in duplicate for three independent productions). Data within a column followed by the same letter are not significantly different according to Duncan's test. Abbreviations: TMM, total mesophilic microorganisms; *L.*, *Lactococcus*; CP, control production inoculated with a commercial starter culture; EP, experimental production inoculated with a commercial starter culture +1% of GPP.

The levels of TMM and *L. lactis* were superimposable for the samples analyzed. The milk inoculated with the reactivated starter culture showed levels of these microorganisms at about 10^7 CFU/mL. After curdling, TMM and *L. lactis* were counted both in control and

experimental curd at 10^8 CFU/g showing an increase of about 1 Log cycle determined as a direct concentration of bacteria due to whey draining [45]. In the final control and experimental cheeses, the levels of TMM and LAB were above 9 Log CFU/g. These data highlighted the ability of the commercial starter culture to drive the fermentation process in presence of 1% (*w/w*) of GPP.

3.4. Identification of Thermoduric Indigenous Milk LAB

Twenty-one presumptive LAB (Gram positive and catalase negative) isolated from pasteurized milk were purified and analysed by RAPD-PCR in order to perform their strain typing. The last analysis, commonly used to discriminate LAB strains isolated from food matrices [46], showed the presence of four different strains (Figure 3).
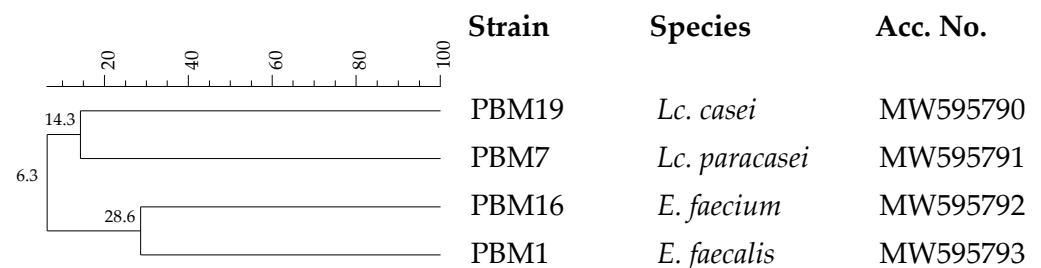
	Strain	Species	Acc. No.
	PBM19	*Lc. casei*	MW595790
	PBM7	*Lc. paracasei*	MW595791
	PBM16	*E. faecium*	MW595792
	PBM1	*E. faecalis*	MW595793

Figure 3. Dendrogram obtained with combined RAPD-PCR patterns generated with three primers for thermoduric indigenous milk LAB strains isolated from pasteurized milk. Abbreviations: *E.*, *Enterococcus*; *Lc.*, *Lacticaseibacillus*.

These strains were identified at the species level by sequencing of the 16S rRNA gene, indicating that the thermoduric indigenous milk LAB included species within the genera *Enterococcus* and *Lactobacillus*. The last genus included two strains belonging to the species *Lacticaseibacillus casei* (PBM19) and *Lacticaseibacillus paracasei* (PBM7) (named *Lactobacillus casei* and *Lactobacillus paracasei*, respectively, before reclassification of Zheng et al. [47]). Regarding the *Enterococcus* genus, one strain was identified as *Enterococcus faecalis* (PBM1), while the strain PBM16 could not be identified at the species level due to the different results of the BLAST and EzTaxon searches. This strain was further analyzed by a species-specific multiplex PCR assay and classified as *Enterococcus faecium*. All the species identified are components of the nonstarter LAB (NSLAB) community implicated in cheese maturation [39], commonly associated with raw milk [48] and cheeses [44,49]. However, their presence in pasteurized milk is mainly due to postpasteurization contamination [50] or to their ability to survive the pasteurization process [51,52].

3.5. Commercial Starter Culture Recognition

In order to evaluate the ability of the commercial starter culture to dominate over the thermoduric indigenous milk LAB and to drive the fermentation process in presence of GPP, a total 101 isolates, collected from inoculated milk to final cheeses, were identified using a polyphasic approach based on microscopic inspection and RAPD-PCR analysis. The results of microscopic inspection confirmed that all isolates were cocci with cells occurring in long chains. The direct comparison of the polymorphic profiles of LAB isolated from pasteurized milk with those of *L. lactis* strains originating from commercial starter LAB, allowed the assessment of the evolution of the added LAB strains (results not shown). This approach confirmed the dominance of the added *L. lactis* strains, originating from commercial LAB starter culture, over thermoduric indigenous milk LAB. Moreover, these data confirmed those obtained by plate counts that showed the ability of commercial starter LAB to drive the fermentation process in presence of GPP.

3.6. Total Phenolic Content

The results of TPC are reported in Figure 4. GPP was characterized by a concentration of TPC of 110.7 g GAE/kg DM, which is higher than the value reported by Harsha et al. [53] for red wine grape pomace of a different Italian cultivar.
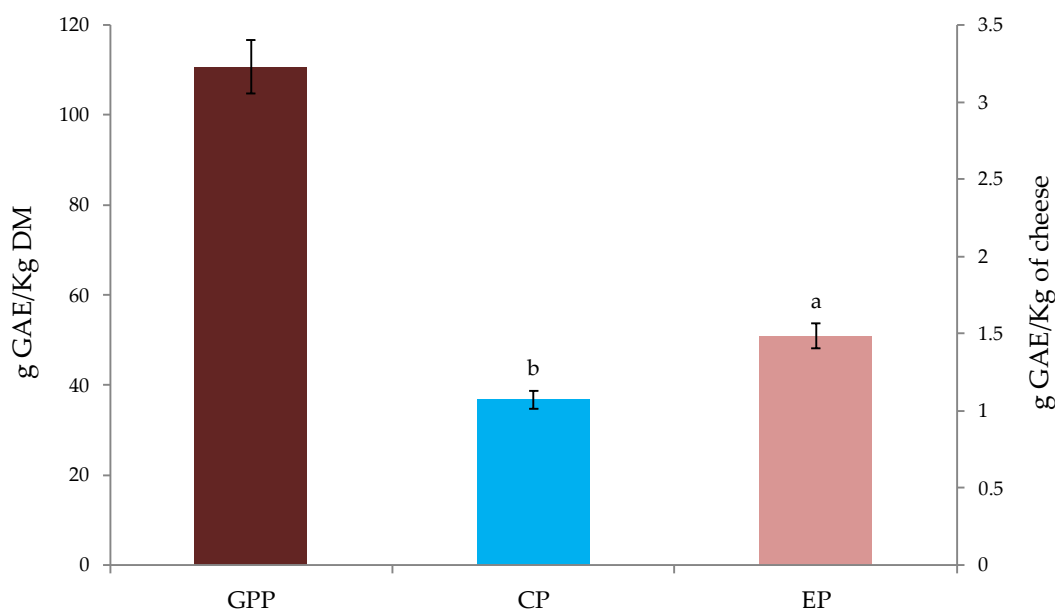

Figure 4. Total polyphenolic content (TPC) of GPP and cheese samples. Units are g GAE/Kg DM for GPP sample and g GAE/Kg for control and experimental cheese samples. Abbreviations: GPP, grape pomace powder; CP, control production inoculated with a commercial starter culture; EP, experimental production inoculated with a commercial starter culture + 1% of GPP. Results indicate mean values and standard deviation of six determinations (carried out in duplicate for three independent productions). Different superscript letters indicate significant differences on TPC according to Duncan's test between control and experimental cheese samples for $p < 0.05$.

To evaluate the effects of GPP addition to cheese, TPC was determined in control and experimental cheese samples. Statistically significant differences ($p < 0.0001$) were found among cheeses. As expected, the amount of TPC detected in control cheese was lower than the corresponding GPP enriched cheeses. In particular, the addition of 1% (w/w) of GPP resulted in an increase of 0.42 g GAE/kg of TPC in experimental cheeses. These results confirmed that the addition of GPP to dairy products results in an increase of TPC [21,23,54].

3.7. Sensory Test

The spider plot reported in Figure 5 shows the sensory analysis of control and experimental cheeses after 30 d of ripening. Except salt, all other sensory attributes evaluated were different between control and experimental cheeses. The highest scores were registered for odor and aroma intensity in experimental cheeses. The scores for sweet, acid perception, fiber sensation, friability, adhesiveness and humidity of GPP-enriched cheeses were higher than those registered in control cheeses. On the contrary, the cheeses enriched with GPP showed lower scores of sweetness and hardness.
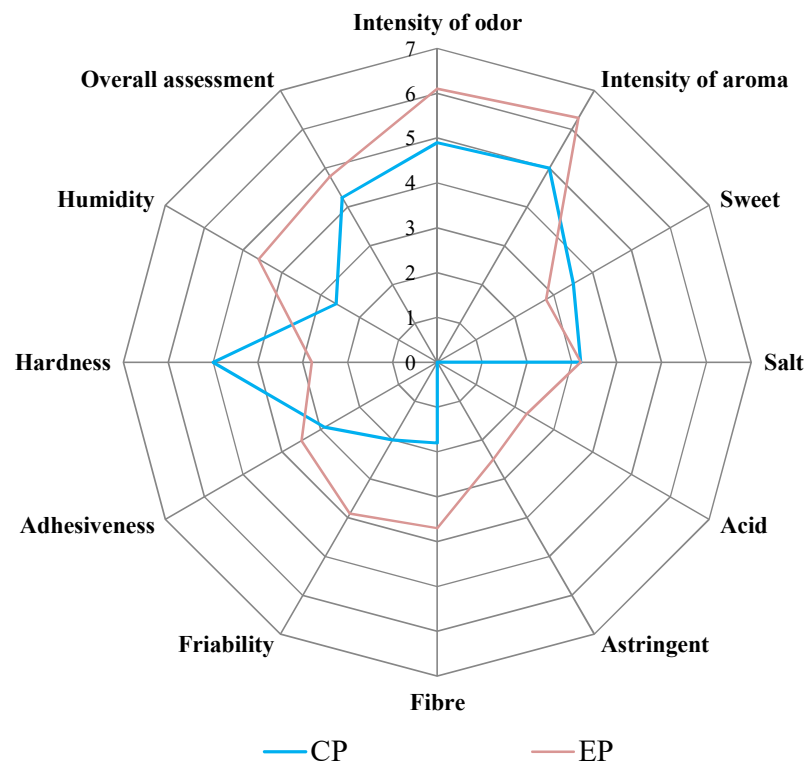
Figure 5. Spider diagrams corresponding to the descriptive sensory analysis of cheeses. Abbreviations: CP, control production inoculated with a commercial starter culture; EP, experimental production inoculated with a commercial starter culture +1% of GPP.

The addition of vegetable by-products to dairy products confirmed the favorable influence on their sensory attributes [20,38,55,56]. As reported by Torri et al. [57], GPP-enriched cheese was described in terms of appearance as marbled with sensations perceived at low intensity of vegetables. However, although the addition of GPP determined large differences in terms of sensory characteristics, the overall assessment of experimental cheese, intended as the degree of overall satisfaction, was higher than the control cheese, indicating a certain appreciation of this GPP-enriched fresh ovine cheese.

4. Conclusions

The obtained results revealed that addition of GPP into ovine cheese at a concentration of 1% (w/w) did not alter the microbiological parameters during fermentation carried out with the commercial starter culture and resulted in a general appreciation by the judges. Furthermore, GPP addition enriched ewe's milk "Primosale" cheese with TPC. This result is of relevance to improve the antioxidant properties of cheeses, even though further studies, such as in vitro gastrointestinal digestion and radical scavenging activities, are necessary to better investigate the beneficial effect of this novel cheese on human health. The present work provided evidence on the industrialization of the process of GPP-enriched cheese making using commercial starter culture.

References

1. Bourgaud, F.; Gravot, A.; Milesi, S.; Gontier, E. Production of plant secondary metabolites: A historical perspective. *Plant Sci.* **2001**, *161*, 839–851. [CrossRef]
2. Maestre, R.; Micol, V.; Funes, L.; Medina, I. Incorporation and Interaction of Grape Seed Extract in Membranes and Relation with Efficacy in Muscle Foods. *J. Agric. Food Chem.* **2010**, *58*, 8365–8374. [CrossRef]
3. Martillanes, S.; Rocha-Pimienta, J.; Cabrera-Bañegil, M.; Martín-Vertedor, D.; Delgado-Adámez, J. Application of Phenolic Compounds for Food Preservation: Food Additive and Active Packaging. In *Phenolic Compounds-Biological Activity*; IntechOpen: London, UK, 2017; pp. 39–58.
4. Yahfoufi, N.; Alsadi, N.; Jambi, M.; Matar, C. The immunomodulatory and anti-inflammatory role of polyphenols. *Nutrients* **2018**, *10*, 1618. [CrossRef] [PubMed]
5. Fontana, A.R.; Antoniolli, A.; Bottini, R. Grape pomace as a sustainable source of bioactive compounds: Extraction, characterization, and biotechnological applications of phenolics. *J. Agric. Food Chem.* **2013**, *61*, 8987–9003. [CrossRef] [PubMed]
6. Gonçalves, E.C.B.A.; Lozano-Sanchez, J.; Gomes, S.; Ferreira, M.S.L.; Cameron, L.C.; Segura-Carretero, A. Byproduct generated during the elaboration process of isotonic beverage as a natural source of bioactive compounds. *J. Food Sci.* **2018**, *83*, 2478–2488. [CrossRef] [PubMed]
7. Pinelo, M.; Arnous, A.; Meyer, A.S. Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release. *Trends Food Sci. Technol.* **2006**, *17*, 579–590. [CrossRef]
8. Dwyer, K.; Hosseinian, F.; Rod, M.R. The market potential of grape waste alternatives. *J. Food Res.* **2014**, *3*, 91. [CrossRef]
9. Acquadro, S.; Appleton, S.; Marengo, A.; Bicchi, C.; Sgorbini, B.; Mandrone, M.; Gai, F.; Peiretti, P.G.; Cagliero, C.; Rubiolo, P. Grapevine green pruning residues as a promising and sustainable source of bioactive phenolic compounds. *Molecules* **2020**, *25*, 464. [CrossRef]
10. Ruggieri, L.; Cadena, E.; Martínez-Blanco, J.; Gasol, C.M.; Rieradevall, J.; Gabarrell, X.; Gea, T.; Sort, X.; Sánchez, A. Recovery of organic wastes in the Spanish wine industry. Technical, economic and environmental analyses of the composting process. *J. Clean. Prod.* **2009**, *17*, 830–838. [CrossRef]
11. Kammerer, D.; Claus, A.; Carle, R.; Schieber, A. Polyphenol screening of pomace from red and white grape varieties (*Vitis vinifera* L.) by HPLC-DAD-MS/MS. *J. Agric. Food Chem.* **2004**, *52*, 4360–4367. [CrossRef]
12. Gómez-Brandón, M.; Lores, M.; Insam, H.; Domínguez, J. Strategies for recycling and valorization of grape marc. *Crit. Rev. Biotechnol.* **2019**, *39*, 437–450. [CrossRef]
13. Baumgärtel, T.; Kluth, H.; Epperlein, K.; Rodehutscord, M. A note on digestibility and energy value for sheep of different grape pomace. *Small Rumin. Res.* **2007**, *67*, 302–306. [CrossRef]
14. Siro, I.; Kápolna, E.; Kápolna, B.; Lugasi, A. Functional food. Product development, marketing and consumer acceptance—A review. *Appetite* **2008**, *51*, 456–467. [CrossRef]
15. Beres, C.; Costa, G.N.; Cabezudo, I.; da Silva-James, N.K.; Teles, A.S.; Cruz, A.P.; Mellinger-Silva, C.; Tonon, R.V.; Cabral, L.M.C.; Freitas, S.P. Towards integral utilization of grape pomace from winemaking process: A review. *Waste Manag.* **2017**, *68*, 581–594. [CrossRef] [PubMed]
16. Tong, X.; Chen, G.C.; Zhang, Z.; Wei, Y.L.; Xu, J.Y.; Qin, L.Q. Cheese consumption and risk of all-cause mortality: A meta-analysis of prospective studies. *Nutrients* **2017**, *9*, 63. [CrossRef]
17. Bachmann, P. Cheese analogues: A review. *Int. Dairy J.* **2001**, *11*, 505–515. [CrossRef]
18. Cutrim, C.S.; Cortez, M.A.S. A review on polyphenols: Classification, beneficial effects and their application in dairy products. *Int. J. Dairy Technol.* **2018**, *71*, 564–578. [CrossRef]
19. Lucera, A.; Costa, C.; Marinelli, V.; Saccotelli, M.A.; Del Nobile, M.A.; Conte, A. Fruit and vegetable by-products to fortify spreadable cheese. *Antioxidants* **2018**, *7*, 61. [CrossRef] [PubMed]

20. Abd Elhamid, A.M. Physicochemical, rheological and sensory properties of Egyptian Kariesh cheese containing wheat bran. *Int. J. Dairy Technol.* **2016**, *69*, 425–432. [CrossRef]

21. Marchiani, R.; Bertolino, M.; Belviso, S.; Giordano, M.; Ghirardello, D.; Torri, L.; Piochi, M.; Zeppa, G. Yogurt enrichment with grape pomace: Effect of grape cultivar on physicochemical, microbiological and sensory properties. *J. Food Qual.* **2016**, *39*, 77–89. [CrossRef]

22. Tseng, A.; Zhao, Y. Wine grape pomace as antioxidant dietary fibre for enhancing nutritional value and improving storability of yogurt and salad dressing. *Food Chem.* **2013**, *138*, 356–365. [CrossRef]

23. Marchiani, R.; Bertolino, M.; Ghirardello, D.; McSweeney, P.L.; Zeppa, G. Physicochemical and nutritional qualities of grape pomace powder-fortified semi-hard cheeses. *J. Food Sci. Technol.* **2016**, *53*, 1585–1596. [CrossRef]

24. Gaglio, R.; Restivo, I.; Barbera, M.; Barbaccia, P.; Ponte, M.; Tesoriere, L.; Bonanno, A.; Attanzio, A.; Di Grigoli, A.; Francesca, N.; et al. Effect on the Antioxidant, Lipoperoxyl Radical Scavenger Capacity, Nutritional, Sensory and Microbiological Traits of an Ovine Stretched Cheese Produced with Grape Pomace Powder Addition. *Antioxidants* **2021**, *10*, 306. [CrossRef]

25. Parente, E.; Cogan, T.M.; Powell, I.B. Starter cultures: General aspect. In *Cheese*; Academic Press: Cambridge, MA, USA, 2017; pp. 201–226.

26. Gregersen, T. Rapid method for distinction of Gram-negative from Gram-positive bacteria. *Eur. J. Appl. Microbiol. Biotechnol.* **1978**, *5*, 123–127. [CrossRef]

27. Koneman, E.W.; Allen, S.D.; Janda, W.M.; Schreckenberger, P.C.; Winn, W.C. The nonfermentative gram-negative bacilli. In *Color Atlas and Textbook of Diagnostic Microbiology*, 7th ed.; Koneman., E.W., Allen, S.D., Janda, W.M., Schreckkenberger, P.C., Winn, W.C., Eds.; Lippincott: Philadelphia, PA, USA, 1997; pp. 253–320.

28. Gaglio, R.; Francesca, N.; Di Gerlando, R.; Mahony, J.; De Martino, S.; Stucchi, C.; Moschetti, G.; Settanni, L. Enteric bacteria of food ice and their survival in alcoholic beverages and soft drinks. *Food Microbiol.* **2017**, *67*, 17–22. [CrossRef]

29. Gaglio, R.; Guarcello, R.; Venturella, G.; Palazzolo, E.; Francesca, N.; Moschetti, G.; Settanni, L.; Saporita, P.; Gargano, M.L. Microbiological, chemical and sensory aspects of bread supplemented with different percentages of the culinary mushroom *Pleurotus eryngii* in powder form. *Int. J. Food Sci. Technol.* **2019**, *54*, 1197–1205. [CrossRef]

30. Liguori, G.; Gentile, C.; Gaglio, R.; Perrone, A.; Guarcello, R.; Francesca, N.; Fretto, S.; Inglese, P.; Settanni, L. Effect of addition of *Opuntia ficus*-indica mucilage on the biological leavening, physical, nutritional, antioxidant and sensory aspects of bread. *J. Biosci. Bioeng.* **2020**, *129*, 184–191. [CrossRef] [PubMed]

31. Cruciata, M.; Gaglio, R.; Scatassa, M.L.; Sala, G.; Cardamone, C.; Palmeri, M.; Moschetti, G.; La Mantia, T.; Settanni, L. Formation and characterization of early bacterial biofilms on different wood typologies applied in dairy production. *Appl. Environ. Microbiol.* **2018**, *84*. [CrossRef] [PubMed]

32. Gaglio, R.; Cruciata, M.; Di Gerlando, R.; Scatassa, M.L.; Mancuso, I.; Sardina, M.T.; Moschetti, G.; Portolano, B.; Settanni, L. Microbial activation of wooden vats used for traditional cheese production and evolution of the neo-formed biofilms. *Appl. Environ. Microbiol.* **2016**, *82*, 585–595. [CrossRef] [PubMed]

33. Altschul, S.F.; Madden, T.L.; Schaffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W. Grapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402. [CrossRef] [PubMed]

34. Chun, J.; Lee, J.H.; Jung, Y.; Kim, M.; Kim, S.; Kim, B.K.; Lim, Y.W. EzTaxon: A web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int. J. Syst. Evol. Microbiol.* **2007**, *57*, 2259–2261. [CrossRef] [PubMed]

35. Jackson, C.R.; Fedorka-Cray, P.J.; Barrett, J.B. Use of a genus- and speciesspecific multiplex PCR for identification of enterococci. *J. Clin. Microbiol.* **2004**, *42*, 3558–3565. [CrossRef] [PubMed]

36. ISO (International Organization for Standardization). *Sensory Analysis e General Guidance for the Design of Test Rooms*; International Standardization Organization (ISO): Geneva, Switzerland, 2007; Volume ISO 8589.

37. Niro, S.; Fratianni, A.; Tremonte, P.; Sorrentino, E.; Tipaldi, L.; Panfili, G.; Coppola, R. Innovative Caciocavallo cheeses made from a mixture of cow milk with ewe or goat milk. *J. Dairy Sci.* **2014**, *97*, 1296–1304. [CrossRef] [PubMed]

38. Costa, C.; Lucera, A.; Marinelli, V.; Del Nobile, M.A.; Conte, A. Influence of different by-products addition on sensory and physicochemical aspects of Primosale cheese. *J. Food Sci. Technol.* **2018**, *55*, 4174–4183. [CrossRef]

39. Faccia, M.; Angiolillo, L.; Mastromatteo, M.; Conte, A.; Del Nobile, M.A. The effect of incorporating calcium lactate in the saline solution on improving the shelf life of fiordilatte cheese. *Int. J. Dairy Technol.* **2013**, *66*, 373–381.

40. Barbaccia, P.; Francesca, N.; Gerlando, R.D.; Busetta, G.; Moschetti, G.; Gaglio, R.; Settanni, L. Biodiversity and dairy traits of indigenous milk lactic acid bacteria grown in presence of the main grape polyphenols. *FEMS Microbiol. Lett.* **2020**, *367*, fnaa066. [CrossRef]

41. Settanni, L.; Moschetti, G. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. *Food Microbiol.* **2010**, *27*, 691–697. [CrossRef]

42. Mainente, F.; Menin, A.; Alberton, A.; Zoccatelli, G.; Rizzi, C. Evaluation of the sensory and physical properties of meat and fish derivatives containing grape pomace powders. *Int. J. Food Sci. Technol.* **2019**, *54*, 952–958. [CrossRef]

43. Rynne, N.M.; Beresford, T.P.; Kelly, A.L.; Guinee, T.P. Effect of milk pasteurisation temperature on age-related changes in lactose metabolism, pH and the growth of non-starter lactic acid bacteria in half-fat Cheddar cheese. *Food Chem.* **2007**, *100*, 375–382. [CrossRef]

44. Gaglio, R.; Gentile, C.; Bonanno, A.; Vintaloro, L.; Perrone, A.; Mazza, F.; Barbaccia, P.; Settanni, L.; Di Grigoli, A. Effect of saffron addition on the microbiological, physicochemical, antioxidant and sensory characteristics of yoghurt. *Int. J. Dairy Technol.* **2019**, *72*, 208–217. [CrossRef]
45. Settanni, L.; Gaglio, R.; Guarcello, R.; Francesca, N.; Carpino, S.; Sannino, C.; Todaro, M. Selected lactic acid bacteria as a hurdle to the microbial spoilage of cheese: Application on a traditional raw ewes' milk cheese. *Int. Dairy J.* **2013**, *32*, 126–132. [CrossRef]
46. Gaglio, R.; Francesca, N.; Di Gerlando, R.; Cruciata, M.; Guarcello, R.; Portolano, B.; Moschetti, G.; Settanni, L. Identification, typing and investigation of the dairy characteristics of lactic acid bacteria isolated from "Vastedda della valle del Belìce" cheeses. *Dairy Sci. Technol.* **2014**, *94*, 157–180. [CrossRef]
47. Zheng, J.; Wittouck, S.; Salvetti, E.; Franz, C.M.; Harris, H.M.; Mattarelli, P.; O'Toole, P.W.; Pot, B.; Vandamme, P.; Walter, J.; et al. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *Int. J. Syst. Evol. Microbiol.* **2020**, *7*, 2782–2858. [CrossRef] [PubMed]
48. Palmeri, M.; Mancuso, I.; Gaglio, R.; Arcuri, L.; Barreca, S.; Barbaccia, P.; Scatassa, M.L. Identification and evaluation of antimicrobial resistance of enterococci isolated from raw ewes' and cows' milk collected in western Sicily: A preliminary investigation. *Ital. J. Food Saf.* **2020**, *9*, 220–225.
49. Corsetti, A.; Caldini, G.; Mastrangelo, M.; Trotta, F.; Valmorri, S.; Cenci, G. Raw milk traditional Italian ewe cheeses as a source of *Lactobacillus casei* strains with acid-bile resistance and antigenotoxic properties. *Int. J. Food Microbiol.* **2008**, *125*, 330–335. [CrossRef] [PubMed]
50. Kagkli, D.M.; Vancanneyt, M.; Hill, C.; Vandamme, P.; Cogan, T.M. *Enterococcus* and *Lactobacillus* contamination of raw milk in a farm dairy environment. *Int. J. Food Microbiol.* **2007**, *114*, 243–251. [CrossRef]
51. De Angelis, M.; Corsetti, A.; Tosti, N.; Rossi, J.; Corbo, M.R.; Gobbetti, M. Characterization of non-starter lactic acid bacteria from Italian ewe cheeses based on phenotypic, genotypic, and cell wall protein analyses. *Appl. Environ. Microbiol.* **2001**, *67*, 2011–2020. [CrossRef] [PubMed]
52. McAuley, C.M.; Gobius, K.S.; Britz, M.L.; Craven, H.M. Heat resistance of thermoduric enterococci isolated from milk. *Int. J. Food Microbiol.* **2012**, *154*, 162–168. [CrossRef]
53. Harsha, P.S.; Gardana, C.; Simonetti, P.; Spigno, G.; Lavelli, V. Characterization of phenolics, in vitro reducing capacity and anti-glycation activity of red grape skins recovered from winemaking by-products. *Bioresour. Technol.* **2013**, *140*, 263–268. [CrossRef]
54. Aiello, F.; Restuccia, D.; Spizzirri, U.G.; Carullo, G.; Leporini, M.; Loizzo, M.R. Improving kefir bioactive properties by functional enrichment with plant and agro-food waste extracts. *Fermentation* **2020**, *6*, 83. [CrossRef]
55. Da Silva, F.D.; Matumoto-Pintro, P.T.; Bazinet, L.; Couillard, C.; Britten, M. Effect of commercial grape extracts on the cheesemaking properties of milk. *J. Dairy Sci.* **2015**, *98*, 1552–1562. [CrossRef] [PubMed]
56. Gaglio, R.; Barbaccia, P.; Barbera, M.; Restivo, I.; Attanzio, A.; Maniaci, G.; Di Grigoli, A.; Francesca, N.; Tesoriere, L.; Bonanno, A.; et al. The Use of Winery by-Products to Enhance the Functional Aspects of the Fresh Ovine "Primosale" Cheese. *Foods* **2021**, *10*, 461. [CrossRef] [PubMed]
57. Torri, L.; Piochi, M.; Marchiani, R.; Zeppa, G.; Dinnella, C.; Monteleone, E. A sensory-and consumer-based approach to optimize cheese enrichment with grape skin powders. *J. Dairy Sci.* **2016**, *99*, 194–204. [CrossRef] [PubMed]