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Physico-Chemical and Sensory Characterization of a Fruit Beer Obtained with the Addition of Cv. Lambrusco Grapes Must

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Abstract: In 2015, Italian Grape Ale (IGA) beers have been included as a new provisional sub-category of special-type fruit beers by the Beer Judge Certification Program, including those products whose brewing process is carried out in presence of determined quantities of grape must. However, information on the effects of these additions on the composition of final beers are still scarce. This work is hence focused on the chromatic, volatile, phenolic and sensory characterization of IGA beers obtained with the addition of grape musts during brewing process. To this aim, different amounts of must (5, 10 and 20%) from cv. Lambrusco red grapes were added to a lager wort before primary fermentation. Beers were then characterized by HPLC-MS, GC-MS and sensory analysis in order to determine phenolic and aroma compounds along with their sensory attributes. Results confirmed the addition of must from cv. Lambrusco grapes capable to enrich beers in color, acids, phenolic (up to 7-folded increased) and volatile compounds, while giving complexity to beers. These results, which were confirmed by a trained sensory panel, are among the very first insights on the impact of red grape must in brewing, both from a compositional and sensory point of view.

Keywords: beer; wine; Lambrusco grape must; IGA; lager; volatile; phenolics

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1. Introduction

Beer is one of the most ancient and widespread consumed beverages in the world. Made mainly from four basic raw materials (malted barley, hops, water and yeast), it is correlated to numerous nutritional properties. A great number of different types or styles of beer are produced. In particular, Lager beer is a classic style that represents more than 90% of brewed beers [1]. Its brewing is carried out by bottom fermenting yeasts at low temperatures (8–15 °C), followed by a storage period in cool conditions (−1–4 °C) in order to stabilize beer and improve its sensory profile. Lager beer is characterized by a pale, golden color with moderate sensory notes of malt and medium-low hop related bitterness [2].

The Beer Judge certification program (https://dev.bjcp.org, accessed on 15 May 2021) lays down the specifications for specialty-type beers obtained by changes of classic styles either by the addition of distinct new ingredients or by developing alternative brewing methods. Namely, fruit beer belongs to the special-type beers elaborated with addition of fruits, such as stone fruit (peach, mango, etc.), pome fruit (e.g., pear, apple), tropical fruit (pineapple, banana) or berries, to obtain a product with a particular sensory profile, with a clear presence of the fruity character but harmonious and balanced with the original base beer [2].

In 2015, a new subcategory of fruit style beer was proposed with the name of Italian Grape Ale (IGA), which is a product resulting from the marriage between beer and wine. It was introduced as a new local style, suitable to enhance the importance of the territory and biodiversity of Italian vineyards, together with the creativity of the brewer [2].

Generally, IGA beers are made with pilsener/pils or other pale base malts with the addition of grapes (or grape must) up to 40% of the grist amount, and used at different brewing steps (boiling, primary or secondary fermentation or bottling). From a sensory point of view, the aromatic influence of the grape should be present without overpowering the base beer profile, while the mouthfeel of the beverage should be characterized by a low to medium body and a slightly higher perception of dryness induced by the grape acidity [2].

The term Lambrusco indicates a large group of red grape varieties used to produce sparkling red wines in Emilia-Romagna, a northern Italian region [3], where those wines represent the first geographical indication in terms of wine volumes (170 million bottles in 2019) and have a strong and recognized linkage with their original territory [4]. Lambrusco wines, which are commonly vinified as rosé or red wines by means of Champenoise or Charmat methods, are characterized by a dark red/purple color; balanced sweetness and acidity; and sweet aromatic notes of fruit, cherries, strawberry or violet [4,5].

Due to its compositive features and the lack of information on derived IGA beers, it seemed interesting to evaluate the impact of cv. Lambrusco grapes must (LGM) additions on the physico-chemical characteristics of a medium-bodied beer.

To this aim, different quantities of cv. Lambrusco grape must were added to a pale Lager malt syrup during the pre-fermentative step of the brewing process. General parameters, phenolic and volatile compounds, and sensory profile were determined on the resulting IGA beer samples, after one month of bottle storage.

To the best of our knowledge, this is the first report on the effects of the use of these Italian grape musts in beer production.

2. Materials and Methods

2.1. Sample Preparation

Lambrusco grape musts (vintage 2020) complying with the "Emilia" geographical Indication product specifications were provided by Caviro Sca (Forlì, Italy).

A brew kit for Lager beer (MrMalt®, Pasian di Prato, Udine, Italy) has been treated according to provider instructions. Briefly, hopped malt syrup was dissolved in 23 L of water (20 °C, fixed residue 160 mg/L) with addition of 1 kg of sucrose and divided into four different batches including a control without any addition (Ctrl), and three experiments with addition of LGM up to 5, 10 and 20%, respectively, of the total volume. Before addition, grape must was heated up to 80 °C in an open stainless steel boiler and rapidly cooled to room temperature in a refrigeration room. Experiments were carried out in two liters laboratory glass fermenters provided of an air trap filled with a SO_2 solution (400 mg/L). Active dry yeasts (Aleaferm Arom, Alea Evolution, Bologna, Italy) were inoculated at 10 g dried yeast to 25 L wort, after rehydration with water at 30 °C and fermented at room temperature (20 °C). Fermentation kinetics was monitored by measuring the weight loss of fermenters. Once weight loss stopped, refermentation was carried out by disposing samples in 250 mL bottles in the presence of 6 g/L of sucrose for two weeks at 20 °C and storage at 4 °C for one month. After storage period, samples were analyzed. All fermentations were carried out by triplicate.

2.2. General Parameters

Titratable acidity and extract were analyzed according to ASBC standards (Beer-8 and wort-2 methods). Volumetric alcohol (v/v) was measured according to International Organization of Vine and Wine (OIV) methods [6]. pH-meter apparatus (Mettler Toledo, Milan, Italy) was used for pH measurements. Astringency was evaluated by means of the

gelatin index [7]. °Brix degree was determined by using a wine refractometer apparatus (Hanna instruments, Padova, Italy).

Optical densities (280, 420, 430, 520, 620 and 700 nm) and CIELAB parameters were determined on degassed and centrifuged (7000 rpm for 10 min) samples in a 1 cm optical path quartz cuvette using a Jasco 810 spectrometer (Tokyo, Japan). EBC color was calculated according to the EBC (European Brewery Convention) method:

$$\text{EBC: } 25.5 * (A430 - A700)$$

where A430 and A700 correspond to the absorbance at 430 and 700 nm, respectively.

CIELAB parameters L* (lightness), a* (redness), b* (yellowness), H (Hue) and C (Chroma) were calculated as described in the OIV methods [6]. Total Polyphenolic Index (TPI) was calculated by spectrophotometric measurements at 280 nm and extrapolation from a previous calibration curve and expressed as Gallic Acid Equivalents (GAE), according to OIV methods [6].

2.3. Free Phenolics Determination by HPLC-ESI-MS/MS

Prior to analysis, beer samples were dilute five times with water, added with 7-OH coumarin (at 5 mg/L) as internal standard and extracted following a modified method of Del Álamo and co-workers [8]. Briefly, solid phase extraction was carried out in a HYPER-SEP RETAIN PEP (Thermo scientific, Waltham, MA, USA) cartridge (60 mg), conditioned with 2 mL of methanol and 2 mL of water. After that, a volume of 10 mL of sample was eluted, and washed with volumes of 2 mL of 0.1% formic acid and 2 mL of 0.1% formic acid + 10% methanol. Finally, elution with 4 mL of acidified methanol (0.1% of formic acid) was carried out. Eluted volume was subsequently concentrated by vacuum centrifuge and redissolved with 2 mL of the HPLC solvent A, before injection.

Analysis of free phenolic compounds by HPLC-ESI-MS/MS was performed in an Agilent 1200 series apparatus (Agilent Technologies, Palo Alto, CA, USA) equipped with an LC-MS detector ion-Trap VL electrospray ionization mass spectrometry system coupled with an Agilent Technologies Chem Station software (version 3.1) for data processing. Separation was performed on a Poroshell 120 SB-C18 HPLC Column 150 lenght × 4.6 mm internal diameter, 2.7 µm particle size (Agilent Technologies) following a method already published [9]. Solvents were water (Solvent A) and acetonitrile (Solvent B) both containing 0.2% of acetic acid. A multi-step gradient sequence was programmed: 0 min, 98% solvent A; 10 min, 95% A; 16 min, 90% A; 21 min, 82% A; 24 min, 80% A; 28 min, 70% A; 31 min, 50% A; 33 min, 0% A; 36 min, 0% A; 37 min, 98% A. Apparatus conditions were: flow 0.8 mL/min, temperature 25 °C, injection volume 50 µL. Detection of phenolics was carried out in both negative and positive modes with the following parameter: N_2 dry gas 11 mL/min, drying temperature 350 °C, nebulizer pressure 65 psi; capillary voltage, −2500 V (positive ionization mode) and +2500 V (negative ionization mode), 600 m/z as target mass, 40% and 100% of compound stability for negative ionization and positive e ionization mode, respectively. Scan range was set at 50–1000 m/z. Chromatograms were also evaluated by using DataAnalysis 4.0 software (Bruker Daltonics, Billerica, MA, USA). Semi-quantification was carried out based on molecular ion peak areas and expressing each compound with respect to the relative area of 7-OH-coumarin (internal standard). Samples were filtered through PVDF filters 0.45 µm before analysis.

2.4. Analysis of Volatile Compounds by GC-MS

Volatile compounds were extracted according to Moio et al. [10]. A 50 mL of two-fold diluted beer with distilled water (1:1), 5 mL of dichloromethane, 250 µL of 2-octanol solution (500 mg/L) as internal standard and 10 g of NaCl were placed in a 60 mL glass bottle. Stream of N_2 was used to remove air before closing. Samples were then disposed in an ice bath for 3 h with continuous stirring at 500 rpm. After phase separation, the organic phase was collected and stored at −30 °C for 12 h before GC-MS analysis.

Analysis was carried out as described by Castro Marin et al., [11] by means of a Trace GC ultra-apparatus coupled with a Trace DSQ mass selective detector (Thermo Fisher Scientific, Milan, Italy) and equipped with a fused silica capillary column Stabilwax DA (Restek, Bellefonte, PA, USA; 30 m, 0.25 mm, i.d., and 0.25 µm film thickness). Helium was used as carrier gas (flow: 1 mL/min).

GC temperature ramp was programmed as follows: from 40 °C (held for 3 min) to 100 °C (held for 1 min) at 3 °C/min, then to 240 °C (held for 10 min) at 5 °C/ min. A volume of 1.5 µL of sample was injected in splitless mode at 250 °C. Electronic Ionization (EI) was used in full scan at 70 eV for detection. Set temperatures for transfer line and ion source were 220 and 260 °C, respectively. Selected mass acquisition range was 30–400 m/z and 1 scan/s of scanning rate.

Identification of compounds was carried out by following a triple criterion: (1) by comparing compound mass spectra and retention time with those of pure standards, (2) matching their respective mass spectra with those present in online libraries Willey and NIST 08, (3) by comparing linear retention index (LRI) calculated under our analytical conditions with already published LRI calculated on polar columns. Quantification was carried out by normalization of integrated areas in total ion current with the area of 2-octanol (internal standard). Analyses were done in duplicate were obtained by using Xcalibur software (version 4.1, Thermo Fisher Scientific, Milan, Italy).

2.5. Sensory Analysis

Sensory analysis was performed at the SensoryLab (Università Cattolica del Sacro Cuore, Piacenza, Italy), a laboratory complying the ISO 8589:2007 standard. Tasting was carried out by a total of ten trained panelists. The samples of Lambrusco IGA beer (Ctrl, 5%, 10% and 20%) were blindly submitted to the panelists and organized through a rotated tasting session. Samples were examined through the Quantitative Descriptive Analysis (QDA), a descriptive analysis technique that includes (i) a qualitative phase in order to generate the descriptors (attributes), (ii) a quantitative phase aimed at evaluating the intensity of the attributes generated, (iii) the statistical processing of the results and their interpretation in order to obtain sensory profiles of the products under examination [12,13]. Selected attributes were:

- Visive: pale yellow, golden yellow, amber, red ruby, ebony, effervescence, fluidity, foam stability, turbidity, foam compactness.
- Olfactive: malty, red fruits, grapes, yeast, fruity, jam, complexity, floral, citrus, vegetable, dimethyl sulfur (DMS), caramel, tropical fruit, spicy, intensity.
- Gustative: malt, astringency, acid, persistence, smoothness, body, sweet.

Attributes were quantified through an unstructured scale from 0 to 10 where 0 indicate the absence of the perception of the sample under examination. Data analysis was carried out through the Sensory package of XLSTAT software (Version 2016, Addinsoft, Paris, France).

2.6. Statistical Analysis

Statistical analysis of physico-chemical dataset and intensity scores from sensory analysis was carried out using the XLSTAT Software package provided of the Sensory tool (Version 2016, Addinsoft, Paris, France).

3. Results and Discussion

3.1. General Parameters

Brewing parameters are outlined in Table 1. Total acidity of musts before primary fermentation increased proportionally with the addition of grape must, causing the consequent pH decrease. This is due to the higher amount of organic acids in LGM which, as already reported in Table 1, had a lower pH (3.40) when compared to Lager beer wort (pH 6.01). After primary fermentation, significant reductions of pH were observed, especially in Ctrl sample (Table 1). As already reported in literature [14,15], this phenomenon is

probably linked to the synthesis of organic acids (mainly succinate, but also malate and citrate to a lesser extent) by yeasts during primary fermentation, as confirmed by the higher titratable acidity, and the production of carbon dioxide (CO_2) as well. For these parameters, data reported no changes after refermentation and storage (Table 1).

Table 1. General parameters of Lager and IGA beers at different brewing steps. TA: Titratable Acidity (g/L); ABV: Alcohol by Volume; Extract (°P); TPI: Total Phenolic Index (mg/L of gallic acid); n.d.: Not determined; # Density of grape must (g/mL). For each brewing phase, in the same column, different letters indicate significant differences according to Tukey's test ($p < 0.05$), $n = 3$.

	pH	TA	ABV %	°Brix	Extract	TPI	Astringency (%)
Pre-ferm							
Grape must	3.40 ^{e}	14.92 ^{a}	-	18.5 ^{a}	1.075 #	1845 ^{a}	-
Beer wort	6.01 ^{a}	0.77 ^{e}	-	8.9 ^{e}	9.02 ^{d}	286 ^{b}	-
5%	4.43 ^{b}	1.25 ^{d}	-	9.3 ^{d}	9.51 ^{c}	304 ^{c}	-
10%	3.98 ^{c}	2.10 ^{c}	-	9.8 ^{c}	9.99 ^{b}	321 ^{d}	-
20%	3.67 ^{d}	3.57 ^{b}	-	10.9 ^{b}	10.56 ^{a}	361 ^{e}	-
1st Ferm							
Ctrl	3.95 ^{a}	2.45 ^{d}	n.d.	-	3.07 ^{a}	275 ^{a}	n.d.
5%	3.77 ^{b}	3.00 ^{c}	n.d.	-	3.07 ^{a}	291 ^{b}	n.d.
10%	3.68 ^{c}	3.67 ^{b}	n.d.	-	3.07 ^{a}	304 ^{c}	n.d.
20%	3.59 ^{d}	4.62 ^{a}	n.d.	-	3.07 ^{a}	345 ^{d}	n.d.
Storage							
Ctrl	3.91 ^{a}	2.43 ^{d}	4.70 ^{d}	-	1.21 ^{b}	274 ^{a}	3.99 ^{d}
5%	3.74 ^{b}	2.80 ^{c}	5.04 ^{c}	-	1.20 ^{a}	291 ^{b}	4.59 ^{c}
10%	3.67 ^{c}	3.13 ^{b}	5.37 ^{b}	-	1.12 ^{a}	304 ^{c}	5.89 ^{b}
20%	3.59 ^{d}	4.87 ^{a}	6.12 ^{a}	-	1.13 ^{a}	338 ^{d}	6.30 ^{a}

Alcohol content of final beers was significantly influenced by the presence of LGM, being the highest (6.12%) for samples supplied with 20% of grape must. These results are supported by °Brix (potential alcohol) measurements of initial musts, where increasing additions of Lambrusco must (18.5 °Brix) raised the potential alcohol parameter in a proportional way.

Similar trend was observed for total polyphenolic index where samples containing more grape must (20%) resulted significantly richer in phenolics (338 mg/L) than Ctrl lager beers (274 mg/L) (Table 1). This tendency could be somehow beneficial since polyphenols contribute to oxidative stability in beers and wines [4,5,16].

As expected, LGM increased the percentage content of phenols reactive to proteins (astringency index) suggesting possible sensory repercussions.

3.2. Color Composition of Lambrusco IGA Beers

3.2.1. EBC Color

Color of beers was first evaluated following the European Brewing Convention method (Table 2) based on the spectrophotometric measurement at wavelengths of 430 and 700 nm. In conventional beers, the first parameter is linked to the wort color due to malt kilning or roasting temperatures, ranging from 2 units for pale beers, up to over 40 for darker malts [16]. As reported in Table 2, EBC color in Ctrl beer ranged from 9.73 in the initial pale wort to 9.15 for finished lager beer. This result was in line with literature [16,17] and corresponded to EBC value indicated by the brew kit producer (6–10 EBC). On the other hand, rising concentrations of grape must (5–20%) led to proportionally higher EBC values, correlated to an increase of the total color. However, EBC color analysis lacks in reliability for redder beers [17] such as, in our experiment, beers produced with the addition of fruit juices. This inaccuracy is due to the absorption of Lambrusco components (e.g., anthocyanins, polyphenols) at wavelengths different from 430 nm. Thus, in order to amend this problem and correctly describe color profile of samples, further color analyses were carried out and will be discussed in the next subsection.

Table 2. Chromatic parameters determined on Lambrusco IGA beers. In the same column, different letters in the same column indicate significant differences according to Tukey's test ($p < 0.05$), $n = 3$. * Color of beer reproduced on the basis of CIELab coordinates by using the Colorizer software (http://colorizer.org, accessed on 15 May 2021).

	420 nm	520 nm	620 nm	EBC	L*	a*	b*	C	H	Color *
Pre-ferm										
Ctrl	0.451 ^{d}	0.128 ^{d}	0.055 ^{c}	9.73 ^{d}	90.97 ^{a}	−0.25 ^{d}	26.63 ^{a}	26.63 ^{b}	90.55 ^{a}	
5%	0.472 ^{c}	0.163 ^{c}	0.055 ^{c}	10.03 ^{c}	88.23 ^{b}	2.77 ^{c}	22.08 ^{b}	22.25 ^{c}	82.84 ^{b}	
10%	0.505 ^{b}	0.293 ^{b}	0.078 ^{b}	11.13 ^{b}	82.69 ^{c}	12.00 ^{b}	19.02 ^{c}	22.51 ^{c}	57.79 ^{c}	
20%	0.705 ^{a}	0.814 ^{a}	0.151 ^{a}	16.4 ^{a}	64.82 ^{d}	38.71 ^{a}	14.85 ^{d}	41.47 ^{a}	20.99 ^{d}	
1st Ferm										
Ctrl	0.392 ^{d}	0.085 ^{d}	0.024 ^{d}	8.31 ^{d}	91.68 ^{a}	−1.37 ^{d}	24.15 ^{a}	24.08 ^{b}	93.27 ^{a}	
5%	0.429 ^{c}	0.154 ^{c}	0.039 ^{c}	9.34 ^{c}	87.87 ^{ab}	3.87 ^{c}	23.12 ^{a}	23.45 ^{b}	80.63 ^{b}	
10%	0.469 ^{b}	0.243 ^{b}	0.055 ^{b}	10.35 ^{b}	80.28 ^{b}	9.93 ^{b}	21.15 ^{b}	23.37 ^{b}	64.86 ^{c}	
20%	0.573 ^{a}	0.506 ^{a}	0.089 ^{a}	13.18 ^{a}	70.80 c	26.37 ^{a}	18.78 ^{c}	32.37 ^{a}	35.46 ^{d}	
Storage										
Ctrl	0.446 ^{b}	0.102 ^{d}	0.03 ^{c}	9.15 ^{d}	92.83 ^{a}	−1.01 ^{d}	25.33 ^{a}	25.35 ^{b}	92.30 ^{a}	
5%	0.464 ^{b}	0.192 ^{c}	0.051 ^{b}	10.5 ^{c}	86.07 ^{b}	5.25 ^{c}	24.38 ^{ab}	25.01 ^{b}	77.84 ^{b}	
10%	0.513 ^{b}	0.287 ^{b}	0.064 ^{b}	11.45 ^{b}	82.70 ^{b}	12.63 ^{b}	23.02 ^{b}	26.23 ^{b}	61.26 ^{c}	
20%	0.675 ^{a}	0.623 ^{a}	0.113 ^{a}	15.8 ^{a}	69.27 ^{c}	31.59 ^{a}	22.41 ^{b}	38.74 ^{a}	35.33 ^{d}	

3.2.2. Spectrophotometric Absorbances at Different Wavelengths: 420, 520 and 620 nm

As pointed out previously, complementary analyses at different wavelengths were carried out with the purpose to provide a detailed description of color of Lambrusco IGA beers. To this aim, according to OIV methods [6], spectrophotometric determinations at 420, 520 and 620 nm were performed (Table 2), corresponding to yellow, red and blue nuances, respectively. In wines, optical densities at 420 nm correspond to the absorbance of flavan-3-ols polymer derivatives, often used as a browning index [18]. On the other hand, absorptions at 520 and 620 nm are linked to the presence of anthocyanins, the principal sources of the color of red wines, with color tones ranging from orange to blue [19].

Before primary fermentation, color densities followed the same trend as the EBC color, with a general increase proportional to grape must addition (Table 2). Data reveal a greater increase of absorbance at 520 nm with LGM addition, suggesting a major contribution of anthocyanins to the total color of IGA beers. After primary fermentation, a slight color diminution was observed in all the samples (Table 2). This decrease corresponded probably to an adsorption of colored compounds by proteins and polysaccharides of yeast cell wall followed by precipitation of lees [20]. Afterward, the final stage of storage led to a slight increase in total color, evidence that could be attributed to chemical oxidation reactions (e.g., browning, polymerization of anthocyanins).

3.2.3. CIELab Parameters (C, H, L*, a*, b*)

A more in-depth and unambiguous adopted method for studying the color of beers was the CIELab method, a tristimulus colorimetry based on the evaluation of the entire visible absorption spectrum of the samples [21]. Data obtained by applying that approach are depicted in Table 2:

- First, the presence of high-colored Lambrusco musts showed a proportional decrease of L parameter, correlated to lightness or presence of white color (Table 2). As a result, it could be inferred that increases in color intensity led to a lower L component, while reductions of color, as occurred after primary alcoholic fermentation were linked to higher L values.
- As expected, when compared to lager beers, coordinates a* (+ red ⇔ green −) and b* (− blue ⇔ yellow +) displaced towards redder and bluer color with grape must addition, reaching the highest a* (31.59) and the lowest b* values (22.41) in beers added with 20% (Table 2). This demonstrated that the presence of anthocyanins from

LGM reduced the yellow component provided by Ctrl pale lager malt (a* = −1.01 and b = 25.33).

- As a consequence, relationships between a* and b* on IGA beers (5, 10 and 20%) shifted the hue (H), generally considered as the "predominant" color, towards reddish hues, proportionally to LGM addition, as showed in Table 2. This result was also confirmed by the color simulation shown in Table 2. Moreover, chroma (C), related to color purity, was also influenced by the presence of grape must, showing the highest (38.74) and the lowest (25.36) values for 20% sample and Ctrl, respectively.

3.3. Phenolics Profiles of Lambrusco IGA Beers

In a first attempt to elucidate the free phenolic composition of final beers, filtered samples were directly injected after 1:2 dilution, and analyzed by LC-MS. Results were substantially unsatisfactory as only 9–15 compounds (depending on the sample considered) were identified (data not shown). The reason for this evidence is certainly the strong suppression of the ionization occurring in the ion source of the ESI system, due to carbohydrates (dextrins and glucans) contained in the matrix [22]. Despite a two-fold sample dilution claimed to conveniently overcome this issue [23], in our analytical and experimental conditions, that procedure was considered not adequate to characterize the beers. Indeed, the pre-treatment of samples by means of a SPE clean-up strategy was extremely beneficial in terms of number of compounds detected and sensitivity, permitting a suitable description of the phenolic diversity among beers.

Following the cited SPE approach, a total of 44 free phenolics were tentatively identified (Table A1), based on (i) both negative and positive MS and MS^2 spectra, (ii) confirmation with pure standards (when available), (iii) elution order in RP-LC similar conditions, (iv) comparison with fragmentation patterns already reported for beer and wines in previous $LC/MS^{(n)}$ experiments.

As expected, because of the limited fragmentation obtained, negative ionization proved to be more sensitive and suited to infer the molecular mass of the aglycones [24], but positive mode was also useful, as it afforded extra certainty, especially in the case of diglycosides or positional isomers (such as compounds nr 24, 27 or 35 of Table A1).

Identified compounds belonged to 7 distinct phenolic classes (Table 3), mostly already reported in beers or red wines (see Table A1 for references) and included simple phenolics such as hydroxybenzoic and hydroxycinnamic acids, flavonoids (either non-glycosylated, mono and diglycosylated), stilbenes and iso-α-acids.

Table 3. Free phenolics quantified in the final beers (mg/L). n.d. = not detected. For each row, different letters indicate significant differences at $p < 0.05$.

Compound	Control	5%	10%	20%
Hydroxybenzoic acids				
Gallic acid	n.d. ^{b}	n.d. ^{b}	0.02 ^{ab}	0.06 ^{a}
Protocatechuic acid-O-hexoside	n.d. ^{b}	0.03 ^{b}	0.10 ^{ab}	0.27 ^{a}
Ethylgallate	0.13	0.34	0.56	0.51
Sum Hydroxybenzoic acids	0.13 ^{b}	0.36 ^{b}	0.68 ^{ab}	0.84 ^{a}
Hydroxycinnamic acids				
Caftaric acid	n.d. ^{c}	0.56 ^{c}	4.62 ^{b}	11.06 ^{a}
2-S-glutathionyl-caffeoyltartaric acid	n.d. ^{b}	n.d. ^{b}	n.d. ^{b}	0.06 ^{a}
t-Coutaric acid	n.d. ^{d}	2.09 ^{c}	17.01 ^{b}	34.52 ^{a}
t-Fertaric acid	n.d. ^{d}	0.70 ^{c}	5.27 ^{b}	18.11 ^{a}
Vanillic acid	0.25	0.21	0.27	0.30
Feruloylquinic acid isomer	2.19 ^{a}	1.21 ^{b}	1.96 ^{ab}	1.84 ^{ab}
t-Caffeic acid	0.10 ^{c}	0.24 ^{c}	0.53 ^{b}	1.20 ^{a}
c-Fertaric acid	n.d. ^{d}	0.54 ^{c}	0.97 ^{b}	1.73 ^{a}
Dihydro-p-coumaric acid	n.d. ^{c}	2.06 ^{b}	5.12 ^{ab}	5.36 ^{a}

Table 3. *Cont.*

Compound	Control	5%	10%	20%
Feruloylquinic acid isomer	1.10	0.79	1.18	1.18
Sinapic acid-O-hexoside	0.88 ^{a}	0.62 ^{b}	0.79 ^{ab}	0.72 ^{ab}
t-Coumaric acid	1.00	0.77	0.86	1.00
t-Ferulic acid	2.73 ^{a}	2.46 ^{b}	2.60 ^{ab}	2.43 ^{b}
Sinapic acid	0.51 ^{a}	0.30 ^{b}	0.40 ^{b}	0.34 ^{b}
Sum Hydroxycinnamic acids	8.77 ^{c}	12.57 ^{c}	41.59 ^{b}	79.84 ^{a}
Flavanols				
(Epi) Catechin hexoside I	0.13 ^{b}	0.23 ^{b}	0.97 ^{a}	1.11 ^{a}
(Epi) Catechin hexoside II	0.21 ^{a}	0.01 ^{b}	n.d. ^{b}	n.d. ^{b}
(Epi) Catechin hexoside III	0.16 ^{a}	0.08 ^{b}	0.06 ^{b}	0.02 ^{b}
Procyanidin dimer (B1)	n.d. ^{d}	0.20 ^{c}	0.73 ^{b}	1.45 ^{a}
Procyanidin dimer	0.10 ^{b}	0.10 ^{b}	0.35 ^{ab}	0.55 ^{a}
(+)-Catechin	1.92 ^{d}	3.31 ^{c}	6.02 ^{b}	10.89 ^{a}
Procyanidin dimer (B2)	n.d. ^{c}	0.07 ^{c}	0.23 ^{b}	0.50 ^{a}
(+)-Epicatechin	n.d. ^{d}	0.48 ^{c}	1.16 ^{b}	2.82 ^{a}
Sum Flavanols	2.51 ^{c}	4.47 ^{bc}	9.51 ^{b}	17.34 ^{a}
Flavones				
Apigenin-C-hexoside-O-hexoside	0.35	0.23	0.26	0.23
Apigenin-C-hexoside-C-pentoside	0.51 ^{a}	0.36 ^{bc}	0.46 ^{ab}	0.28 ^{c}
Apigenin C-hexoside (Isovitexin)	0.41 ^{a}	0.33 ^{a}	0.40 ^{a}	n.d. ^{b}
Sum Flavones	1.27 ^{a}	0.91 ^{ab}	1.12 ^{a}	0.51 ^{b}
Flavonols				
Myricetin 3-glucoside	n.d. ^{c}	0.15 ^{b}	0.42 ^{ab}	1.01 ^{a}
Quercetin-3-glucuronide	n.d. ^{d}	0.33 ^{c}	1.15 ^{b}	2.55 ^{a}
Laricitrin 3-glucoside	n.d. ^{c}	n.d. ^{c}	0.09 ^{b}	0.20 ^{a}
Quercetin	n.d. ^{c}	0.14 ^{c}	6.50 ^{a}	1.88 ^{b}
Sum Flavonols	0.00 ^{c}	0.62 ^{b}	8.15 ^{a}	5.63 ^{a}
Stilbenes				
t-Resveratrol glucoside	n.d. ^{b}	n.d. ^{b}	0.08 ^{b}	1.05 ^{a}
c-Resveratrol glucoside	n.d. ^{c}	n.d. ^{c}	0.12 ^{b}	0.40 ^{a}
t-Resveratrol	n.d. ^{c}	0.21 ^{b}	0.41 ^{ab}	0.71 ^{a}
Sum Stilbenes	0.00 ^{d}	0.21 ^{c}	0.60 ^{b}	2.17 ^{a}
Others				
Tyrosol	3.53 ^{a}	2.58 ^{ab}	2.89 ^{ab}	2.20 ^{b}
Indole lactic acid glucoside	n.d. ^{d}	0.33 ^{c}	0.81 ^{b}	1.52 ^{a}
Dihydroquercetin 3-O-rhamnoside (Astilbin)	n.d. ^{d}	0.78 ^{c}	1.91 ^{b}	3.58 ^{a}
Dihydromyricetin 3-O-rhamnoside	n.d. ^{d}	1.28 ^{c}	2.57 ^{b}	4.26 ^{a}
Sum Others	3.53 ^{d}	4.97 ^{c}	8.17 ^{b}	11.57 ^{a}
Sum phenolics	16.21 ^{c}	24.11 ^{c}	69.83 ^{b}	117.89 ^{a}

The addition of grape must to the beers contributed to enrich the phenolic profile both qualitatively and quantitatively (Table 3). Despite being reported in some beers or in hop [25] flavonols, flavanonols (e.g., dihydroquercetin and dihydromyricetin glycosides) and stilbenes, which are common constituents of grapes [26], were only found in IGA samples while other chemical classes such as phenolic acids and flavanols were considerably enhanced in content after progressive addition of cv. Lambrusco grape must.

Hydroxycinnamic acids were the most abundant and diverse phenolic class in samples (Table 3). As expected, for Ctrl beers, ferulic acid and its glycosides were largely predominant [25,27] representing more than 50% of the total amount. However, after grape must addition, other tartaric esters of coumaric, caffeic and ferulic acids were found to prevail to a great extent.

Flavanols amounts increased up to 6-fold after incremental additions of grape must and, considering their sensory features, this may influence astringency and bitterness of

the products. In addition, IGA beers contained a significative amount of stilbenes (mainly as resveratrol glycosides) which may enhance the dietary value of those beers [26].

Flavone glycosides, two (epi)catechin hexosides and iso-α-acids were the only phenolics that significantly reduced their content in 20% samples. These compounds exclusively derive from malt and hop, respectively [25], and undergo a sort of dilution with the increase of LGM percentages. It is worth to mention that the satisfactory quantitation of iso-α-acids was hampered by the poor chromatographic separation. Due to this, their amount has not been reported in Table 3.

Overall, by adding cv. Lambrusco grape must, final products had up to a 7-fold increase in the total phenolic content, in this way positively influencing the nutritional and antioxidant value of the final product.

3.4. Volatile Composition of Lambrusco IGA Beers

The GC/MS analysis of beers volatiles permitted the identification of about 90 compounds, which will be commented as separate chemical classes (Table A2).

3.4.1. Alcohols

As reported in Figure 1, concentrations of volatile alcohols did not show any significant difference among samples as a sum. However, if single compounds are considered, a proportional increase of C_6-alcohols such as n-hexanol and cis-3-hexen-1-ol, corresponding to the presence of LGM can be observed (Table A2). Among others, C_6 alcohols are responsible for the varietal herbaceous character of grapes [28], highlighting a relationship with the presence of grape must.

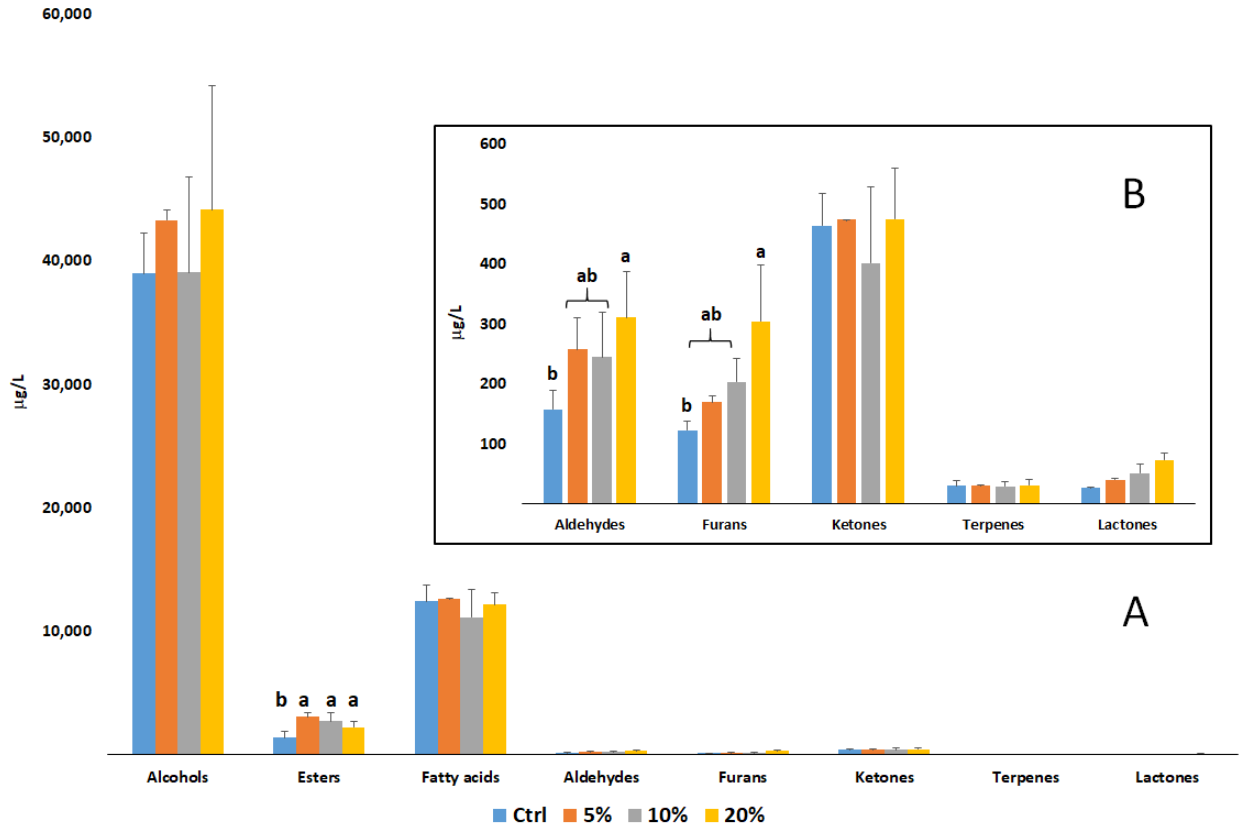

Figure 1. Concentration (µg/L) of quantified volatile compounds grouped by chemical classes. (**A**): Alcohols, esters, fatty acids. (**B**): Aldehydes, furans, ketones, terpenes, lactones (shown in the inset for sake of clarity, due to low amounts). For each chemical class, different letters indicate significant differences according to Tukey's test, $n = 3$.

Table A2 also reports in 20% IGA samples a lesser content of 4-vinyl guaiacol (4VG) which, depending on the beers style and the concentration, is sometimes considered undesirable [29]. According to literature, 4VG is synthesized by yeasts or, during high temperature mashing treatments, after decarboxylation of free ferulic acid, an hydroxycinnamate deriving from the husk and endosperm of cereals [29]. However, up to 90% of ferulic acid in malt is bound to polysaccharides which need to undergo enzymic hydrolytic cleavage during brewing and fermentation before to release the acid in the free form [30]. Therefore, the lower presence of 4VG in 20% Lambrusco IGA samples probably depends on the diminution of bound ferulic acid in wort because of the addition of grape must, and the subsequent reduced amount of ferulic acid to be used by yeasts for the synthesis of that volatile phenol.

3.4.2. Esters

If compared to Ctrl beer, all IGA samples had significant higher content of esters as a sum (Figure 1). Additions of grape must influence the overall ester concentrations in a significant way. This could be attributed to the higher content of easily fermentable sugars, such as glucose and fructose in those worts that drove to a higher production of esters (especially the ethyl congeners) [31]. Their presence could positively affect the aroma of finished product since volatiles such as isoamyl acetate, ethyl hexanoate and ethyl octanoate (Table A1) have been associated with banana and sweet and sour apple notes, respectively [32].

3.4.3. Vicinal Diketones (VDKs)

The presence of VDKs (such diacetyl and 2,3 pentanedione) above their odor threshold in beer (from 0.1 to 1 mg/L depending on the product [33]) is generally regarded as a defect, since their flavor, described as buttery or butterscotch-like, is undesirable in many beer styles.

In our samples, only traces of VDK 2,3-pentanedione were quantified in 20% IGA samples (Table A2). Its reduction product, 3-hydroxy-2-pentanone was however identified, showing a content which increased with the addition of grape must. On the other hand, acetoin and 2,3-butanediol, both deriving from the reduction of diacetyl acted by yeasts [34], were also quantified, following a trend similar to 3-hydroxy-2-pentanone (Table A2). This evidence could be due to the lower pH of samples added of LGM (Table 1) that may have boosted the enzymatic reduction of VDKs precursors during fermentation [35], hence generating higher amounts of derived alcohols (Table A2). Furthermore, it has been demonstrated that faster fermentation kinetics, as is the case of IGA samples at 10 and 20% (Figure 2), increase the synthesis of valine, leucine or isoleucine by yeasts, and the consequent accumulation of VDKs [36] which, at pH < 4, would promptly be enzymatically reduced to their respective diols.

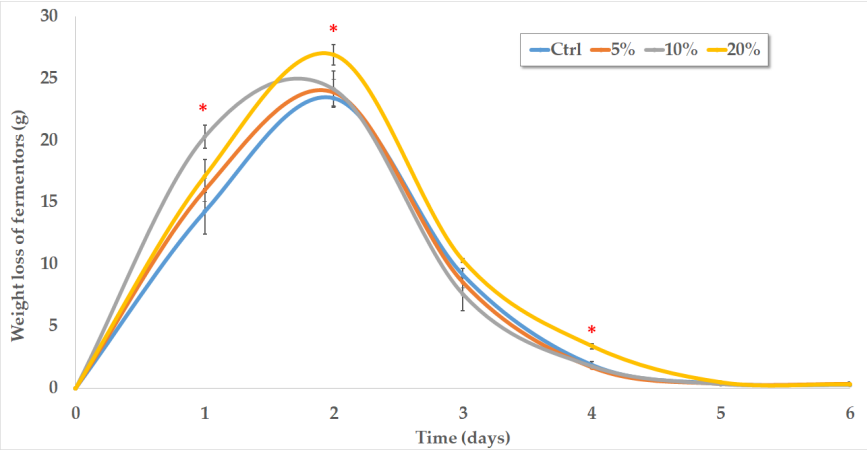

Figure 2. Fermentation kinetics of beer samples as obtained by the weight loss of fermenters.

3.4.4. Furans, Pyrans and Lactones

These heterocyclic aromatic molecules occur in beverages and foods rich in sugars, in particular, when submitted to heating treatments such as boiling or toasting [37], in some cases providing sweet or toasted aromas [38]. As depicted in Table A2, furans were absent or at very low concentration in Ctrl Lager beers, according with the low kilning temperatures used for pale lager malts, not conducive to high generation of those molecules [39]. However, a progressive increase of furans with grape must addition was observed (Figure 1), reaching the highest content in beers spiked with 20% of LGM (Table A2). These results suggested that pasteurization of grape must prior to pre-fermentative addition triggered further Maillard and caramelization reactions, generating compounds not present in beer wort, such as 5-ethyldihydro-2-(3H)-furanone, 2H-pyran-2,6-(3H)-dione and 5-hydroxymethyldihydrofuran-2-one and promoting the increase of those already formed during malting.

Similar trend was observed for furfural derivatives (furfural, furfuryl alcohol and HMF) as outlined in Table A2. As previously reported [40], these compounds are originated from pentoses and hexoses (such as glucose and fructose) during pasteurization of LGM, contributing notably to the sensory attributes of food or beverages subjected to high temperatures such as beer.

3.4.5. Terpenes

Terpenes may originate from both hops and grapes and play an important role in brewing and winemaking since their presence influence the overall flavor of the product [41]. Only five terpenes were identified (Table A2). This is somewhat in line with reported low hop aroma of Lager beers [1]. Moreover, the heat-treatments given to the grape musts may have led to the loss of constitutive terpenes. When considering the whole class of compounds (Figure 1), no significant differences were reported among samples. However, Table A2 provides information on the origin of distinct compounds, such as epoxylinalool oxide and (+)-1-menthene, only present on IGA beers, coming from Lambrusco grapes. In contrast, geranial concentration was reduced with additions of grape musts, suggesting a dilution phenomenon of this compound present in the initial beer wort.

3.5. Sensory Analysis

The sensory features of beers were assessed by means of QDA sessions whose results were then statistically treated to determine the discriminatory power possessed by each sensory descriptor selected by the panelists. In Figure 3, *p*-values (on Y axis) lower than 0.05 indicate a significant overall capacity of the relative descriptor to discriminate the samples.

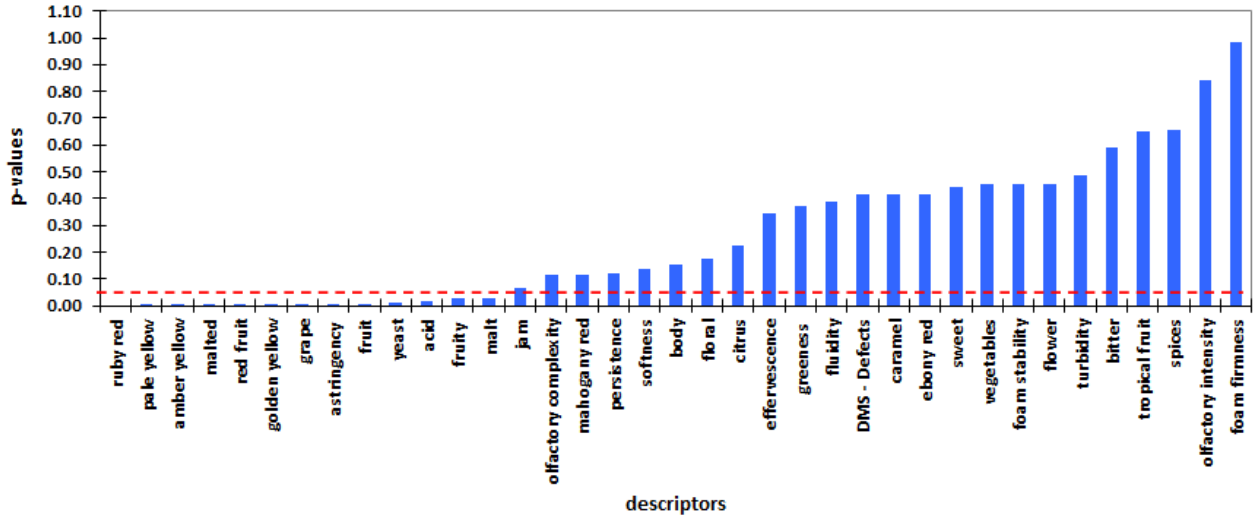

Figure 3. Overall discriminatory power of the sensory descriptors, as expressed by the ANOVA *p*-values. The dotted red line represents the limit for significance at $p \leq 0.05$.

Ruby red, pale yellow, amber and golden yellow colors showed the greatest discriminatory power among visual parameters. This evidence is in accordance with analytical parameters outlined in Table 2 where increasing addition of red must generated a significant difference among samples, mainly due to the presence of anthocyanins in grapes as confirmed by spectrophotometric absorbances at 520 nm (Table 1).

The olfactory descriptors with the greatest discriminatory impact were malt, red fruit, fruity, grape and yeast character, certainly related to the progressive dilution of the beer wort with LGM.

Astringency and acidity were discriminatory as well, depending on the greater or lesser content of tannins and organic acids due to the grape musts, as already reported in Table 1. Other sensory attributes such as foamability, olfactory intensity or body did not appear to be influenced by any LGM additions to beer wort.

Figure 4 shows the QDA elaborated for every single beer, as described by the relevance of each descriptor in discriminating that sample with respect to the others. In that Figure, colorized histograms indicate parameters possessing significant discrimination power. Ctrl lager beer (Figure 4A) was characterized by pale and golden yellow color to which corresponded the absence of amber and ruby color correlated to LGM addition. Further, lager beer lacked sensory notes related to grape musts such as fruitiness, grape and red fruits aroma (Figure 4A), while it had the highest malt character attributed to the starting lager must. It was also described as the least acid and astringent.

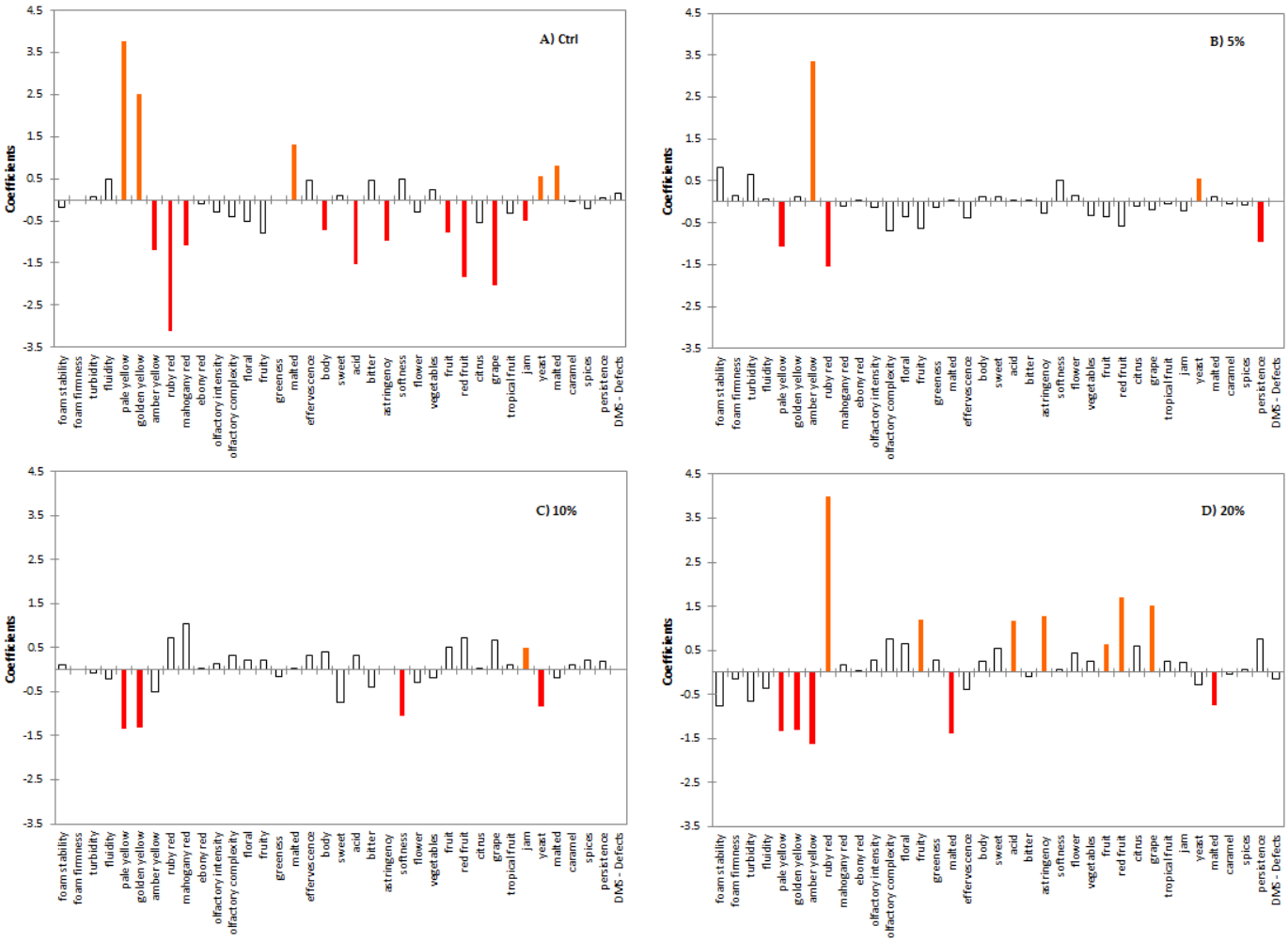

Figure 4. Discriminatory power of the sensory descriptors for each beer sample (**A**–**D**). Colorized histograms indicate significant discrimination at $p \leq 0.05$.

For sample with 5% of grape must added (Figure 4B), the descriptor with the grater discriminatory power was the presence of yellow amber color, further confirmed by the loss of pale-yellow color. Panelists indicated a lower gustatory persistence but a recognizable yeast reminiscence. Addition of 10% LGM was characterized by the lower presence of pale and amber color (Figure 4C), while the sample containing 20% of grape must presented a greater ruby red color which was the most important discriminating descriptor (Figure 4D). Contrarily to the IGA at 5 and 10%, the addition of 20% LGM significantly impacted on both olfactory and gustative descriptors, being described as more astringent, fruity and acidic but low in malt notes.

4. Conclusions

Overall, if compared to Ctrl lagers, beer samples produced by pre-fermentative addition of cv. Lambrusco grape musts at 5, 10 and 20%, presented a greater complexity. The most influenced parameter was the color, which changed towards redder shades, as suggested by spectrophotometric determination and confirmed by sensory analysis. On the other hand, liquid and gas chromatography analysis revealed a general increase of phenolics (up to 7-fold) and volatile components in IGA samples, including compounds beneficial for the overall nutritional value (such as stilbenes) and shelf life of beers. The highest addition of grape must (20%) led to the diminution of some malt notes, thus reducing the beer character and emphasizing some wine-derived sensory descriptors. This experiment successfully allowed the elaboration and characterization of 4 distinct products, from pale yellow to ruby hues, with progressively different aromas and acidities that could find an interesting space in the brewing market. Based on the above results, a further matter being investigated by our group is the antioxidant and antiradical properties of IGA beers and the addition of musts coming from distinct Italian aromatic white grapes such as cv. Moscato or Pignoletto grapes.

Author Contributions: Conceptualization, A.C.M. and F.C.; methodology, A.C.M., G.M., M.L. and F.C.; investigation, A.C.M., F.B., E.R. and G.M.; resources, F.C.; data curation, A.C.M., F.B. and E.R.; writing—original draft preparation, A.C.M., M.L. and F.C.; writing—review and editing, F.C.; supervision, F.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Free phenolic compounds tentatively identified in at least one of the samples. MS^2 fragments in both negative and positive modes are given in order of abundance. n.d. = not detected; * identification confirmed with pure standards. For those compounds lacking of pure standards, identification, based on fragmentation patterns in either negative or positive mode, was tentatively assigned according to [(1)] Cheiran et al., 2019 [42]; [(2)] Quifer-Rada et al., 2015 [43]; [(3)] Pati et al., 2014 [44]; [(4)] Flamini, 2013 [25].

Compound	Class	Compound	rt (min.)	(M-H)	MS^2 (−)	$(M + H)^+$	MS^2 (+)
1	OH-benzoic acids	Gallic acid *	6.68	169	125	n.d.	n.d.
2	OH-benzoic acids	Protocatechuic acid-O-hexoside [(1,2)]	9.90	315	153, 109	n.d.	n.d.
4	OH-cinnamic acids	Caftaric acid [(3)]	13.3	311	177, 149	n.d.	n.d.
5	OH-cinnamic acids	2-S-glutathionyl-caffeoyltartaric acid [(3)]	14.7	616	484, 440, 272	618	543, 489, 264
6	Flavanols	(Epi) Catechin hexoside I [(1,2)]	16.8	451	289, 161	n.d.	n.d.
8	OH-cinnamic acids	Coutaric acid [(3)]	18.1	295	163, 149	n.d.	n.d.
9	Others	Tyrosol *	18.5	137	119	n.d.	n.d.

Table A1. *Cont.*

Compound	Class	Compound	rt (min.)	(M-H)	MS^2 (−)	$(M + H)^+$	MS^2 (+)
10	Flavanols	(Epi) Catechin hexoside II [1,2]	18.6	451	289	n.d.	n.d.
11	Flavanols	(Epi) Catechin hexoside III [1,2]	20.4	451	289	n.d.	n.d.
12	Flavanols	Procyanidin dimer (B1) *	20.4	577	425	579	427, 409, 291
13	Flavanols	Procyanidin dimer [3,4]	20.8	577	425, 407	579	427, 409, 453, 291
14	OH-cinnamic acids	*t*-Fertaric acid [3,4]	21.2	325	193	n.d.	n.d.
15	Flavanols	(+)-Catechin *	21.7	289	245, 179, 203	291	123, 139, 165, 273
16	OH-cinnamic acids	Vanillic acid *	22.2	167	123, 152, 108	n.d.	n.d.
17	OH-cinnamic acids	Feruloylquinic acid isomer [1,2]	22.3	367	193, 173	n.d.	n.d.
18	OH-cinnamic acids	*t*-Caffeic acid *	23.1	179	135	n.d.	n.d.
19	OH-cinnamic acids	*c*-Fertaric acid [3,4]	23.1	325	235, 265	n.d.	n.d.
20	OH-benzoic acids	Ethylgallate *	23.6	197	182, 153	n.d.	n.d.
21	Flavanols	Procyanidins dimer (B2) *	23.7	577	425, 407	579	427, 409, 291
22	Flavanols	(+)-Epicatechin *	24.5	289	245, 179, 203	291	
23	Others	Indole lactic acid glucoside [3]	25.4	366	204, 186, 142	n.d.	n.d.
24	Flavones	Apigenin-C-hexoside-O-hexoside [1,2]	25.6	593	312	595	577, 433, 415, 367
25	OH-cinnamic acids	Dihydro-*p*-coumaric acid [3]	25.7	165	147	n.d.	n.d.
26	OH-cinnamic acids	Feruloylquinic acid isomer [1,2]	25.8	367	193, 173	n.d.	n.d.
27	Flavones	Apigenin-C-glycoside-C-pentoside [1,2]	26.0	563	443, 473, 383, 545	565	547, 499, 529, 481, 469
28	OH-cinnamic acids	Sinapic acid-*O*-hexoside [1,2]	26.1	385	267	n.d.	n.d.
29	Flavonols	Dihydromyricetin 3-*O*-rhamnoside [4]	26.4	465	339, 301, 447	467	449, 431, 321
30	Flavonols	Myricetin glucuronide [4]	26.5	493	317	n.d.	n.d.
31	OH-cinnamic acids	*t*-Coumaric acid *	26.6	163	119	n.d.	n.d.
32	Flavonols	Myricetin-3-glucoside *	26.6	479	317	n.d.	n.d.
33	Stilbenes	*t*-Resveratrol glucoside [4]	28.4	389	227	n.d.	n.d.
34	OH-cinnamic acids	*t*-Ferulic acid *	28.4	193	134, 149, 178	n.d.	n.d.
35	Flavones	Apigenin-6C-glucoside (Isovitexin) [1,2]	28.5	431	311, 341	433	367, 415, 337, 313, 283
36	OH-cinnamic acids	Sinapic acid [2]	28.8	223	208, 164	n.d.	n.d.
37	Flavonols	Quercetin-3-glucuronide [4]	29.0	477	301	479	303, 317
38	Flavonols	Laricitrin-3-glucoside [4]	29.2	493	331	495	333
39	Others	Dihydroquercetin 3-O-rhamnoside (Astilbin) [4]	29.5	449	303, 285	451	415, 433, 315
40	Stilbenes	*c*-Resveratrol glucoside [4]	31.1	389	227	n.d.	n.d.
41	Stilbenes	*t*-Resveratrol *	32.6	227	185	n.d.	n.d.
42	Flavonols	Quercetin *	33.4	301	179	n.d.	n.d.
43	Isoacids	Iso-α-cohumulone [2]	36.9	347	251, 329	n.d.	n.d.
44	Isoacids	Iso-α-ad/n-humulone [2]	37.3	361	265, 343	n.d.	n.d.

Table A2. Quantification (μg/L) of volatile compounds of beer samples after storage period. In the same column, different letters indicate significant differences obtained through Tukey's test ($p < 0.05$), $n = 3$.

	Ctrl	5%	10%	20%
Alcohols				
Isobutyl alcohol	2156.02 ^{a}	3039.43 ^{a}	2706.57 ^{a}	2729.83 ^{a}
n-Butanol	22.66 ^{b}	43.67 ^{ab}	43.64 ^{ab}	60.62 ^{a}
2-penten-4-ol	21.71 ^{a}	36.62 ^{a}	26.10 ^{a}	28.74 ^{a}
Isoamyl alcohol	18,396.81 ^{a}	22,120.79 ^{a}	20,515.96 ^{a}	23,002.98 ^{a}
3-methyl-3-Buten-1-ol	13.33 ^{a}	18.62 ^{a}	16.41 ^{a}	14.85 ^{a}

Table A2. *Cont.*

	Ctrl	5%	10%	20%
2-Hexanol	18.79 [a]	25.46 [a]	19.06 [a]	22.41 [a]
4-Methyl-1-pentanol	21.12 [b]	35.74 [a]	31.40 [ab]	31.67 [ab]
2-Buten-1-ol, 3-methyl-	4.56 [a]	4.35 [a]	4.88 [a]	3.33 [a]
3-Methyl-1-pentanol	22.99 [b]	41.65 [a]	39.93 [a]	46.15 [a]
meso-3,4-Hexanediol	2.28 [b]	4.27 [ab]	5.58 [ab]	19.11 [a]
n-hexanol	6.33 [c]	33.01 [b]	46.96 [b]	77.94 [a]
3-ethoxy-1-Propanol	36.41 [a]	40.07 [a]	32.05 [a]	25.70 [a]
cis-3-Hexene-1-ol	n.d. [c]	4.06 [bc]	7.71 [b]	15.45 [a]
2-Butoxyethanol	5.28 [a]	4.93 [a]	4.39 [a]	5.20 [a]
n-Heptanol	44.87 [a]	53.71 [a]	53.59 [a]	46.26 [a]
2-Methyl-4-octanol	7.60 [a]	7.64 [a]	7.86 [a]	10.37 [a]
3-methyl-2-Octanol	6.03 [a]	6.26 [a]	6.91 [a]	5.38 [b]
3-Ethyl-2-heptanol	7.29 [a]	7.66 [a]	8.57 [a]	7.20 [a]
2-Ethylhexanol	9.25 [a]	11.45 [a]	11.84 [a]	10.39 [a]
3-Nonanol	20.59 [a]	21.37 [a]	23.41 [a]	18.86 [a]
2-Nonanol	3.78 [a]	2.71 [a]	2.52 [a]	1.10 [a]
2-3-Butanediol	229.66 [a]	223.91 [a]	236.56 [a]	335.82 [a]
1-Octanol	20.60 [a]	26.75 [a]	24.88 [a]	20.39 [a]
2,3-Butanediol	54.89 [a]	47.58 [a]	51.99 [a]	84.17 [a]
1,2-Propanediol	11.32 [a]	7.44 [a]	9.31 [a]	13.73 [a]
1-Methoxy-2-butanol	12.77 [a]	12.26 [a]	12.26 [a]	14.52 [a]
n-decanol	6.36 [a]	9.70 [a]	7.63 [a]	7.11 [a]
2,7-dimethyl-4,5-Octanediol	17.30 [a]	21.11 [a]	16.72 [a]	20.29 [a]
Benzyl alcohol	10.41 [b]	22.89 [b]	10.25 [b]	51.69 [a]
Phenetyl alcohol	17,725.51 [a]	17,315.69 [a]	15,020.83 [a]	17,356.69 [a]
2-Methoxy-4-vinylphenol	30.53 [a]	34.95 [a]	23.74 [ab]	15.71 [b]
1-Heptadecanol	41.36 [a]	26.26 [a]	31.21 [a]	34.44 [a]
total alcohol	38,988.39 [a]	43,311.99 [a]	39,060.74 [a]	44,142.52 [a]
Fatty Acids				
Propanoic acid	12.97 [a]	18.39 [a]	17.09 [a]	15.75 [a]
Isobutyric acid	112.83 [a]	138.58 [a]	117.95 [a]	95.45 [a]
Butanoic acid	52.38 [a]	82.91 [a]	86.69 [a]	85.86 [a]
pentanoic acid	352.55 [a]	371.95 [a]	308.84 [a]	268.45 [a]
Hexanoic acid	2314.31 [a]	2351.84 [a]	2070.12 [a]	2327.57 [a]
(E)-2-methyl-2-Pentenoic acid	68.41 [a]	59.23 [a]	53.70 [a]	44.74 [a]
Octanoic acid	4929.90 [a]	5037.27 [a]	4369.91 [a]	5279.22 [a]
Nonanoic acid	28.23 [ab]	31.94 [a]	15.34 [b]	25.15 [ab]
Decanoic acid	2619.96 [a]	2427.08 [a]	2226.25 [a]	2470.79 [a]
9-Decenoic acid	344.72 [a]	440.73 [a]	303.29 [a]	384.66 [a]
Benzoic acid	104.46 [a]	128.05 [a]	127.18 [a]	111.61 [a]
Dodecanoic acid	643.58 [a]	622.25 [a]	496.97 [a]	454.87 [a]
Phenylacetic acid	215.05 [a]	257.18 [a]	221.23 [a]	229.06 [a]
Phenylpropionic acid	29.41 [b]	35.59 [ab]	42.86 [a]	29.03 [b]
Tetradecanoic acid	47.15 [a]	48.55 [a]	48.98 [a]	32.73 [a]
Pentadecanoic acid	14.61 [a]	17.34 [a]	19.79 [a]	14.34 [a]
(Z)-Cinnamic acid	93.62 [a]	87.22 [a]	70.27 [a]	61.57 [a]
Hexadecanoic acid	259.66 [ab]	335.39 [a]	351.29 [a]	157.31 [b]
Octadecanoic acid	179.57 [a]	179.02 [a]	179.86 [a]	68.99 [a]
total acids	12,423.40 [a]	12,670.50 [a]	11,127.62 [a]	12,157.13 [a]
Esters				
Isoamyl acetate	109.29 [b]	943.53 [a]	863.25 [a]	483.38 [a]
Ethyl hexanoate	35.90 [b]	379.97 [a]	338.96 [a]	167.04 [a]

Table A2. *Cont.*

	Ctrl	5%	10%	20%
1-Hexyl acetate	n.d. [b]	5.83 [a]	7.84 [a]	8.86 [a]
Hex-4-enoic acid, ethyl ester	2.00 [ab]	4.29 [a]	4.45 [a]	n.d. [b]
Ethyl lactate	28.56 [b]	55.14 [ab]	64.49 [ab]	112.68 [a]
Ethyl caprylate	60.52 [b]	440.66 [a]	369.12 [a]	210.39 [a]
Ethyl 3-hydroxybutyrate	5.22 [b]	7.31 [b]	10.78 [ab]	23.03 [a]
Ethyl 2-hydroxycaproate	n.d. [b]	n.d. [b]	3.26 [a]	3.80 [a]
Ethyl decanoate	24.24 [a]	69.99 [a]	82.93 [a]	34.99 [a]
Diethyl succinate	n.d. [c]	4.32 [b]	6.86 [ab]	10.55 [a]
ethyl 9-decenoate	3.74 [a]	15.08 [a]	12.31 [a]	7.82 [a]
β-Phenylethyl acetate	753.74 [a]	873.51 [a]	622.85 [a]	660.97 [a]
Ethyl dodecanoate	15.97 [a]	15.73 [a]	20.27 [a]	11.35 [a]
N-Acetylglycine ethyl ester	8.33 [b]	9.87 [b]	33.19 [a]	43.23 [a]
Ethyl hydrogen succinate	130.62 [c]	237.11 [b]	271.71 [b]	441.85 [a]
total esters	1409.52 [b]	3062.34 [a]	2712.28 [a]	2219.95 [a]
Furans				
Furfural	n.d. [b]	2.70 [ab]	3.77 [a]	6.26 [a]
2.4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	6.89 [b]	17.55 [ab]	21.48 [a]	18.25 [ab]
5,5-dimethylfuran-2(5H)-one	11.53 [a]	0.00 [b]	0.00 [b]	0.00 [b]
Furfuryl alcohol	60.07 [b]	88.17 [b]	94.89 [ab]	151.60 [a]
2(3H)-Furanone, 5-ethyldihydro-	n.d. [b]	n.d. [b]	3.41 [a]	4.89 [a]
4H-Pyran-4-one, 3-hydroxy-2-methyl	25.46 [a]	28.61 [a]	28.50 [a]	22.39 [a]
2H-Pyran-2,6(3H)-dione	n.d. [b]	n.d. [b]	10.17 [a]	13.93 [a]
2(3H)-Furanone, dihydro-5-pentyl	8.25 [a]	13.68 [a]	12.97 [a]	12.84 [a]
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	10.46 [a]	12.71 [a]	12.21 [a]	11.95 [a]
5-Hydroxymethyldihydrofuran-2-one	n.d. [b]	n.d. [b]	8.50 [a]	53.03 [a]
HMF	n.d. [b]	6.78 [a]	8.22 [a]	9.63 [a]
total furans	122.66 [b]	170.20 [ab]	204.12 [ab]	304.76 [a]
Ketones				
2,3-Pentanedione	n.d. [b]	n.d. [b]	n.d. [b]	6.37 [a]
Acetoin	29.95 [c]	68.16 [b]	40.52 [bc]	127.71 [a]
3-hydroxy-2-pentanone	n.d. [c]	4.23 [b]	3.78 [b]	14.99 [a]
4-Octanone, 5-hydroxy-2,7-dimethyl-	42.22 [a]	54.66 [a]	52.36 [a]	61.74 [a]
4-hydroxy-2-butanone	359.99 [a]	313.60 [a]	272.58 [a]	238.60 [a]
3-hydroxy-4-phenyl-2-butanone	32.43 [a]	37.58 [a]	36.89 [a]	40.80 [a]
total ketones	464.59 [a]	474.00 [a]	402.35 [a]	475.21 [a]
Aldehydes				
Benzeneacetaldehyde	158.16 [a]	194.44 [a]	177.38 [a]	206.68 [a]
p-Hydroxybenzaldehyde	n.d. [b]	63.14 [ab]	67.30 [ab]	104.66 [a]
total aldehydes	158.16 [b]	257.58 [ab]	244.68 [ab]	311.34 [a]
Terpenes				
Epoxy Linalool oxide	n.d. [b]	n.d. [b]	n.d. [b]	4.25 [a]
Geranial	5.25 [a]	4.94 [a]	3.23 [ab]	1.58 [b]
B-citronellol	2.54 [a]	3.13 [a]	3.53 [a]	2.04 [a]
(+)-1-Menthene	n.d. [c]	n.d. [c]	2.13 [b]	5.75 [a]
Farnesol	22.32 [a]	22.47 [a]	20.10 [a]	17.85 [a]
total terpenes	30.11 [a]	30.54 [a]	28.99 [a]	31.47 [a]
Lactones				
Butyrolactone	15.63 [c]	29.68 [bc]	39.10 [b]	67.50 [a]
y-Dodecalactone	10.62 [a]	10.57 [a]	11.89 [a]	5.64 [a]
total lactones	26.25 [c]	40.25 [b]	50.99 [ab]	73.14 [a]
Others				
Scyllo-inositol	n.d. [c]	2.50 [b]	3.85 [ab]	7.98 [a]
Methionol	736.93 [a]	838.61 [a]	684.14 [ab]	941.54 [a]
Acetylpyrrole	13.70 [a]	16.20 [a]	13.83 [a]	12.59 [a]
Acetovanillone	n.d. [c]	13.13 [b]	14.70 [b]	27.80 [a]

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