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Gluten reduction in beer by hydrodynamic cavitation assisted brewing of barley malts

Lorenzo Albanese [a], Rosaria Ciriminna [b], Francesco Meneguzzo [a, *], Mario Pagliaro [b, **]

[a] Istituto di Biometeorologia, CNR, via G. Caproni 8, 50145 Firenze, Italy
[b] Istituto per lo Studio dei Materiali Nanostrutturati, CNR, via U. La Malfa 153, 90146 Palermo, Italy

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ABSTRACT

While gluten content in beers can be quite toxic to coeliac patients as well as to the broader group of gluten-intolerant people, using gluten-free raw ingredients leads to severe deprivation of flavor and taste, as well as other existing methods to lower the gluten concentration are still generally not firmly established as well as quite costly. During the development and test of a novel brewing technology based on controlled hydrodynamic cavitation, early evidence arose of gluten reduction in wort and finished beer from 100% barley malt, in correspondence with suitable cavitation regimes during both mashing and fermentation. Experimental tests are reviewed and discussed, while few hypotheses are advanced, pointing to the degradation of proline residues, the most recalcitrant among gluten constituents, leading to gluten concentration reduction in the unfermented wort and/or during fermentation and maturation, the latter due to the enhanced proline assimilation by yeasts. Direction for further research includes at least repetition of experiments and design of new ones, extension of the range of cavitation regimes, and identification of strict operational parameters as functions of brewing recipes.

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1. Introduction

With nearly 200 billion liters per year, beer is the alcoholic beverage most widely consumed around the world (Amienyo & Azapagic, 2016). Its basic ingredients, *i.e.* water, malt or grains, hops and yeasts, and production methods have barely changed over centuries beyond obvious technological improvements and ingredients diversification (Ambrosi, Cardozo, & Tessaro, 2014; Pires & Brányik, 2015), while knowledge of the respective microbiological processes is well established (Bokulich & Bamforth, 2013).

Despite a moderate dietary beer consumption is considered a healthy attitude under certain conditions (de Gaetano et al., 2016), the gluten content, arising from barley and wheat malts and grains from which most beers are produced, make that beverage unsuitable for consumption by coeliac disease patients (Hager, Taylor, Waters, & Arendt, 2014).

Contrary to most other inflammatory disorders, both genetic precursors and exogenous environmental factors triggering the coeliac disease are known since long enough, along with its basic mechanisms (Sollid, 2002). It develops in susceptible patients because of their intolerance to ingested fractions of cereal proteins referred collectively as gluten, including proteins of barley (hordein), wheat (gliadin) and rye (secalin). In particular, the gluten epitopes recognized by the immune system in the human intestine are generally very rich in proline and glutamine residues, which are amino acids and gluten components. Proline residues, showing high levels in barley (Deželak, Zarnkow, Becker, & Košir, 2014; Malalgoda & Simsek, 2016), were observed to play a key role by means of their multiple ways of influencing the immunogenicity of gluten peptides (Balakireva & Zamyatnin, 2016).

Production and marketing of *very low gluten content* (<100 mg/L) or *gluten-free* (<20 mg/L) beers is still in its starting phase and the projected market value in Europe is estimated on the order of several billion Euros per year (Harasym & Podeszwa, 2015).

Most gluten-free beers foresees the use of at least a fraction of malts derived from cereals and pseudo-cereals not containing gluten or its precursors, such as sorghum, buckwheat, quinoa, amaranth (Wijngaard & Arendt, 2006; de Meo et al., 2011), maize and oat (Yeo & Liu, 2014). Nevertheless, the respective brewing techniques for cereals different from barley have not yet been well

Abbreviations: BIAB, Brew In A Bag; CN, Cavitation Number; FAN, Free Amino-Nitrogen; GRAS, Generally Recognized As Safe; HC, Hydrodynamic Cavitation; SG, Silica Gel.

* Corresponding author.
** Corresponding author.
E-mail addresses: f.meneguzzo@ibimet.cnr.it (F. Meneguzzo), mario.pagliaro@cnr.it (M. Pagliaro).

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established, despite some recent encouraging results (Mayer et al., 2016).

Alternatively, generally complex and costly techniques are sometimes used, such as filtration and enzymatic ones, aimed at conditioning the malts in order to boost the processes leading to the precipitation of proteins, in particular polypeptides, during mashing, fermentation and possibly stabilization (Dostálek, Hochel, Méndez, Hernando, & Gabrovská, 2006; Hager et al., 2014). Beyond uncertainties, complexity and costs, finished beers most often fall far away from traditional aroma and flavor customers are used to. An alternative technique consists in the use of silica gel (SG) at some stage of the brewing process, mainly fermentation, in order to selectively remove proteins, without practically affecting both valuable yeast nutrients such as free amino-nitrogen (FAN) and foam-causing proteins (Benítez, Acquisgrana, Peruchena, Sosa, & Lozano, 2016). Although SG is recognized as a safe food additive both in US and Europe, its use adds to cost and process complexity.

Fermentation, usually lasting several days since the pitching of yeast strains in the cooled and aerated wort, is the most important brewing step for the gluten reduction in traditional beers. During fermentation, assimilation of fermentable sugars, amino acids, minerals and other nutrients occurs along with metabolic production of ethanol, CO_2, higher alcohols, esters and other substances (Bokulich & Bamforth, 2013; Landaud, Latrille, & Corrieu, 2001; Pires & Bányik, 2015). In particular, amino acids accumulated in the fermenting wort supply nearly all the nitrogen needed by the yeasts' cellular biosynthesis in the form of FAN, as well as affect bitterness, flavor and foam stability (Choi, Ahn, Kim, Han, & Kim, 2015). Among amino acids, most important is glutamine, a gluten component, along with other ones belonging to the so called "Group A" which undergo the fastest assimilation by yeasts' cells at a rate depending on the specific yeast strain (Pires & Bányik, 2015): glutamine assimilation and transformation explains the fall of gluten concentration during fermentation. Once Group A amino acids are assimilated, other ones belonging to Groups B and C are more gradually and slowly assimilated until nitrogen-deprived residuals from original amino acids are turned into higher (fusel) alcohols and esters, strongly impacting beers' flavor. An only amino acid belongs to Group D, namely proline, whose assimilation by yeast cells was deemed negligible until few years ago (Lekkas, Stewart, Hill, Taidi, & Hodgson, 2005). However, more recently the proline itself, whose concentration in the fermenting wort can be quite high, was found to lead to the formation of fusel alcohols, therefore impacting beer's aroma, flavor and overall alcohol content (Procopio, Krause, Hofmann, & Becker, 2013). The proline assimilation rate revealed a high sensitivity to the yeast strain, increasing in high stress conditions due to the shortage of more easily assimilated amino acids, as well as a positive dependence on the availability of molecular oxygen, which is a scarce resource during anaerobic fermentation.

Given the fast assimilation of glutamine, practically the gluten concentration in the wort as well as in the finished beer will depend on the proline assimilation rate, which, along with its role about gluten toxicity, makes its assimilation, degradation and further reduction during fermentation and maturation—the latter lasting several weeks either in dedicated vessels or in bottles —very beneficial to the food safety of the finished beers.

This study shows early evidence of the potential for brewing of conventional barley malts assisted by controlled hydrodynamic cavitation (HC) to reduce the gluten concentration in the respective beers by means of suitable cavitation regimes and operational parameters, *i.e.* by purely electro-mechanical means, without either changing ingredients or using additives as well as any other technological pathway.

2. Materials and methods

2.1. Brewing unit

A dedicated equipment was built from known or commonly available commercial components, in order to investigate the effects of hydrodynamic cavitation processes upon gluten concentration.

Fig. 1 shows the experimental installation, including a closed hydraulic loop with total volume capacity around 230 L, powered by a centrifugal pump (Lowara, Vicenza, Italy, model ESHE 50–160/75 with 7.5 kW nominal mechanical power) with open impeller 0.174 m in diameter. Rotation speed was set around 2900 rpm. As shown in the manufacturer's technical documentation at page 48 ("Serie e-SH (in Italian), "2016), the maximum pressure and volumetric flow rate were around 4 atm and 1500 L min^{-1}, respectively.

A Venturi tube, with the same geometry described in detail in Fig. 2(B) of a previous study by the authors (Albanese, Ciriminna, Meneguzzo, & Pagliaro, 2015), is used as the cavitation reactor and preferred over an orifice plate since it was observed that orifices are quickly obstructed by the circulating solid particles.

The design allows for upscaling of a single installation unit up to the order of 10,000 L, for housing further pumps and cavitation reactors, and for straightforward integration of isolated components, such as pumps and HC reactors, into existing brewing and fermentation plants of virtually any size.

All but one of the tests designed to study the HC effects upon the gluten concentration ran in brew-in-the-bag (BIAB) mode, where the malts are not allowed to circulate, being caged in the cylindrical vessel shown in Fig. 1, in turn made up of a stainless steel fine grid with a perforated pipe arranged along the vessel axis, connected to the same external pump used for thermal stabilization. In BIAB tests, malt milling before mashing was required and performed by means of a small semiautomatic stainless steel roller mill. On the contrary, hops—being pitched after the removal of the cylindrical

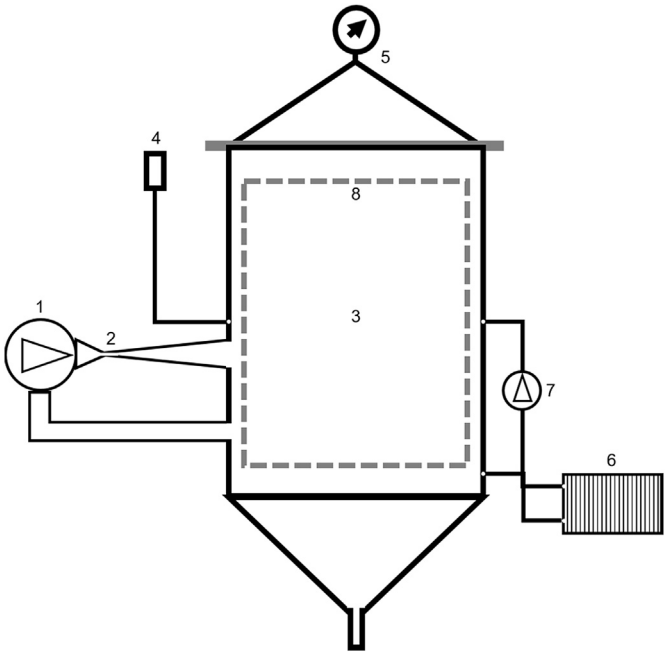

Fig. 1. Simplified scheme of the experimental HC-based installation. 1—centrifugal pump, 2—HC reactor, 3—main vessel, 4—pressure release valve, 5—cover and pressure gauge, 6—heat exchanger, 7—circulation pump, 8—malts caging vessel. Other components are commonly used in state-of-the-art hydraulic constructions.

vessel—were allowed to circulate.

Further details can be found in to section 2.1 of the authors' previous study (Albanese, Ciriminna, Meneguzzo, & Pagliaro, 2017).

2.2. Hydrodynamic cavitation

Physics and chemistry of controlled HC in liquid media have received increasing coverage by the scientific literature, paralleling the growing industrial applications (Carpenter et al., 2016; Ciriminna, Albanese, Meneguzzo, & Pagliaro, 2017; Šarc, Stepišnik-Perdih, Petkovšek, & Dular, 2017). In particular, HC-assisted beer brewing has recently been investigated by means of a real-scale experimental and demonstration device (Albanese et al., 2017), finding a potential for successful industrial developments, along with significant advantages and no apparent drawbacks. Dramatic reduction of saccharification temperature, increased and accelerated peak starch extraction, significant reduction of operational time after traditional stages, such as dry milling and boiling, are made unessential, represent the most important benefits, along with relevant energy saving, shorter cleaning time, volumetric heating which prevents caramelization and overall simplification of both structural setup and operational management of brewing processes. No cavitational damage to the equipment as well as no wort and beer oxidation showed up after thousands hours of operation.

In HC-assisted beer brewing, the choice of hydrodynamic cavitation over the acoustic one was explained based on the HC's outperformance under virtually any aspect, namely energy efficiency, cavitational yield and scalability, so much that, in the authors' opinion, the debate about cavitation technology selection for industrial applications is basically over. As well, Venturi-type stationary reactors were identified as the most appealing candidates for industrial-scale applications, outperforming both rotating rectors and stationary orifice plates, among the others (Albanese et al., 2017, 2015; Ciriminna et al., 2017).

HC regimes are practically identified according to the values assumed by a single dimensionless parameter, *i.e.* the cavitation number (CN, also indicated as σ in the following), shown in its simplest form in Equation (1).

$$\sigma = (P_0 - P_v) \Big/ \left(0.5\rho u^2\right) \quad (1)$$

where P_0 (Nm^{-2}) is the average pressure measured downstream of a cavitation reactor, such as a Venturi tube or an orifice plate, where cavitation bubbles collapse, P_v (Nm^{-2}) is the liquid vapor pressure (a function of the average temperature for any given liquid), ρ (kgm^{-3}) is the liquid density, and u (ms^{-1}) is the flow velocity measured through the nozzle of the cavitation reactor.

For the scope of this study, only the developed cavitation with $0.1<\sigma<1$ will be considered (Albanese et al., 2017; Bagal & Gogate, 2014; Gogate, 2002).

While severe limitations about such an oversimplified description of the HC regimes were recently highlighted (Šarc et al., 2017), the detailed description of the experimental equipment, and the adherence of the cavitation reactor's geometry to the recommendations of the latter authors, were shown to allow the above classical description (Albanese et al., 2017).

Recently, a sample of beer wort after yeast pitching was treated in an ultrasonic bath of given frequency and variable power (Choi et al., 2015). In particular, the observed acceleration of the FAN utilization by yeasts, in turn affecting the beer's organoleptic and physiological properties, could be interpreted in terms of the yeasts' enhanced assimilation of proline. Although achieved by means of ultrasound-assisted cavitation in laboratory-scale experiments, those early results provided a useful guidance for real-scale HC applications.

2.3. Analytical instruments and methods

Along with thermometer and manometer sensors onboard the main production unit, few specialized off-line instruments were used to measure the chemical and physiological properties of wort and beer, relevant to this study.

The acidity was measured by means of pH-meter (Hanna

Table 1
Beer production tests, ingredients and conditions.

Test ID	Brewing unit[a]	Net volume (L)	Malt	Cavitating malts	Hops[b]	Added sugars[d]	Fermentation[e]
CO1	1(A)	186	Pilsner 25 kg Cara Pils 1.6 kg Cara Hell 2.6 kg Weizen 2 kg	Yes	Perle 0.1 kg Hers[c] 0.3 kg Saaz 0.2 kg	W 0.96 kg (bot)	Standard
C2	1(B)	170	Pilsner 25 kg Cara Pils 1.6 kg Cara Hell 2.6 kg Weizen 2 kg	No	Perle 0.1 kg Hers 0.4 kg Saaz 0.1 kg	W 0.96 kg (bot)	Standard
C5	1(B)	170	Pilsner 25 kg Cara Pils 3.6 kg Cara Hell 2.6 kg	No	Perle 0.1 kg Hers 0.3 kg Saaz 0.2 kg	W 10 kg (fer) W 0.96 kg (bot)	Standard
C6	1(B)	170	Pilsner 25 kg Cara Pils 3.6 kg Cara Hell 2.6 kg	No	Perle 0.3 kg Hers 0.4 kg Saaz 0.2 kg	W 8 kg (fer) W 0.96 kg (bot)	Standard
C7	1(B)	170	Pilsner 28.5 kg Cara Pils 2.5 kg	No	Perle 0.6 kg Saaz 0.5 kg	W 0.84 kg (bot)	Standard
C8	1(B)	170	Pale 26 kg Cara Pils 3 kg	No	Perle 0.2 kg Hers 0.1 kg	B 1.0 kg (fer) W 0.96 kg (bot)	Device
C9	1(B)	170	Pale 26 kg Cara Pils 3 kg	No	Perle 0.2 kg Hers 0.1 kg	B 1.0 kg (fer) W 0.96 kg (bot)	Device
C10	1(B)	170	Pale 26 kg Cara Pils 3 kg	No	Perle 0.2 kg Hers 0.1 kg	B 1.0 kg (fer) W 0.96 kg (bot)	Device

[a] With reference to Fig. 1: 1(A) = circulating malts (no caging vessel); 1(B) = with caging vessel (BIAB mode).
[b] Hops cavitating in any test.
[c] Hers = Hallertau Hersbrucker hop.
[d] W = simple white sugar; B = candied brown sugar; bot = before bottling; fer = before fermentation.
[e] Standard = fermentation in cylinder-conical vessel 200 l. Device = fermentation performed into the experimental device.

Instruments, Padova, Italy, model HI 99151) with automatic pH calibration and temperature compensation. The sugar concentration in the wort during mashing and before fermentation was measured in Brix percentage degrees by means of a refractometer (Hanna Instruments, Padova, Italy, model HI 96811, scaled from 0% to 50% Brix, resolution 0.1%, precision ±0.2% in the 0–80 °C temperature range, and automatic temperature compensation in the 0–40 °C range). Brix readings were then converted to starch extraction efficiency (Bohačenko, Chmelík, & Psota, 2006).

Physico-chemical and physiological parameters of fermenting wort and finished beer were measured by means of a 6-channel photometric device (CDR, Firenze, Italy, model BeerLab Touch). Specific to this study was the free amino-nitrogen, or FAN (30–300 mg/L, resolution 1 mg/L). Reagents were of analytical grade.

The gluten concentration measurement method was RIDASCREEN® Gliadin competitive, *i.e.* the official standard method for gluten determination according to the Codex Alimentarius (Hager et al., 2014; Rallabhandi, Sharma, Pereira, & Williams, 2015). The results were in units mg/L with an upper limit at 270 mg/L and the measurement uncertainties, as declared by the accredited laboratory in charge of the analyses, were equal to 6.7% for results above 150 mg/L, as low as 2.4% below such threshold.

The microbiological measurement, *i.e.* the counting of the alive

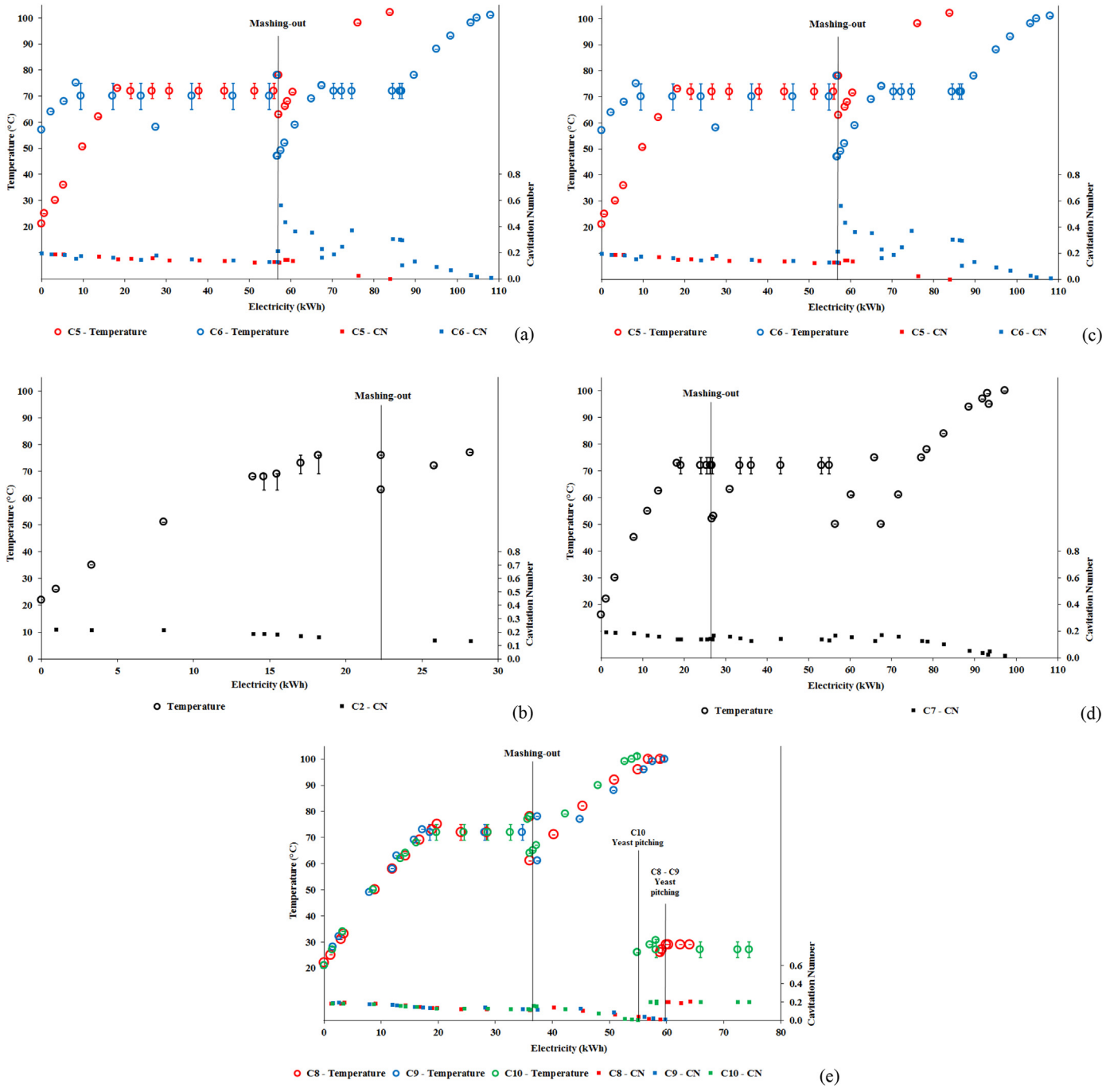

Fig. 2. Description of brewing processes in terms of temperature with respective uncertainties and cavitation number, as functions of the consumed electricity, for tests CO1 (a), C2 (b), C5 and C6 (c), C7 (d), C8, C9 and C10 (e).

yeast cells, was performed according to the method explained in the Appendix to one of authors' previous studies (Albanese et al., 2015).

2.4. Brewing ingredients

Pilsner or Pale were used as the base barley malts in all the performed tests, along with smaller fractions of Cara Pils, Cara Hell and Weizen, the latter ones supporting body, flavor, aroma and foam stability of the finished beer.

Among the hops, different combinations of pelletized German Perle, Saaz and German Hersbrucker were used. In the course of few tests, candied brown sugar was added to the wort before fermentation, while regular white sugar was added to the fermented wort before bottling and maturation. Finally, fermentation was activated by means of the dry yeast strain Safale US-05, requiring temperature between 15 °C and 24 °C and maximum alcohol content 9.2%, used in any test in the identical proportion of 67 g per 100 L.

2.5. Brewing tests

Few basic features of the brewing tests carried out in this study are summarized in Table 1. No simple sugar was added during the mashing stage in any test. Test CO1 was performed with cavitating grains, all the others in BIAB mode. Three tests (C8, C9 and C10) were performed with exactly the same ingredients and, contrary to the others, the respective wort fermented into the same processing device shown in Fig. 1.

The brewing processes are summarized in Fig. 2(a–e), in terms of temperature and cavitation number against consumed energy. In tests CO1, C2, C5, C6, C7 and C9, the wort underwent cavitation processes only before yeast pitching, while HC was activated also just after yeast pitching in tests C8 and C10.

3. Results

3.1. Overall results

The primary objectives of the HC-assisted brewing experiments were the demonstration of the technological feasibility, the identification of the respective advantages over traditional processes, and the proof of technological scalability up to the industrial level (Albanese et al., 2017). The finding that the gluten concentration in the produced beers can be substantially reduced by means of suitably tuning the HC regimes represents a further benefit that, to the best knowledge of the authors, can represent another significant novelty.

Fig. 3 shows the gluten concentration for all the performed tests, measured at different times starting at the beginning of fermentation and during maturation in bottles. Although barley malts were used practically in the same proportion to the respective volumes, as apparent from Table 1, differences are striking, with tests C6, C8, C9, C10, and partially test C7, showing far less final gluten concentration than the others. Given the relative uncertainties mentioned in Section 2.3., as well as the fact that all gluten measurements were performed in triplicate, always falling into the respective uncertainty ranges, the differences between the two groups of data values are significant.

3.2. Effects of cavitation before yeast pitching

In tests CO1 (with cavitating malts) and C2, using the same malts mix, no additional hydraulic pressure was applied, resulting in cavitation numbers between 0.1 and 0.2 (lower at the highest temperatures in test CO1), and the electricity consumptions were equal to 39 kWh (CO1) and 28 kWh (C2, terminated at the temperature of 78 °C), as shown in Fig. 2(a) and (b).

Fig. 2 shows that the gluten concentrations in the beers

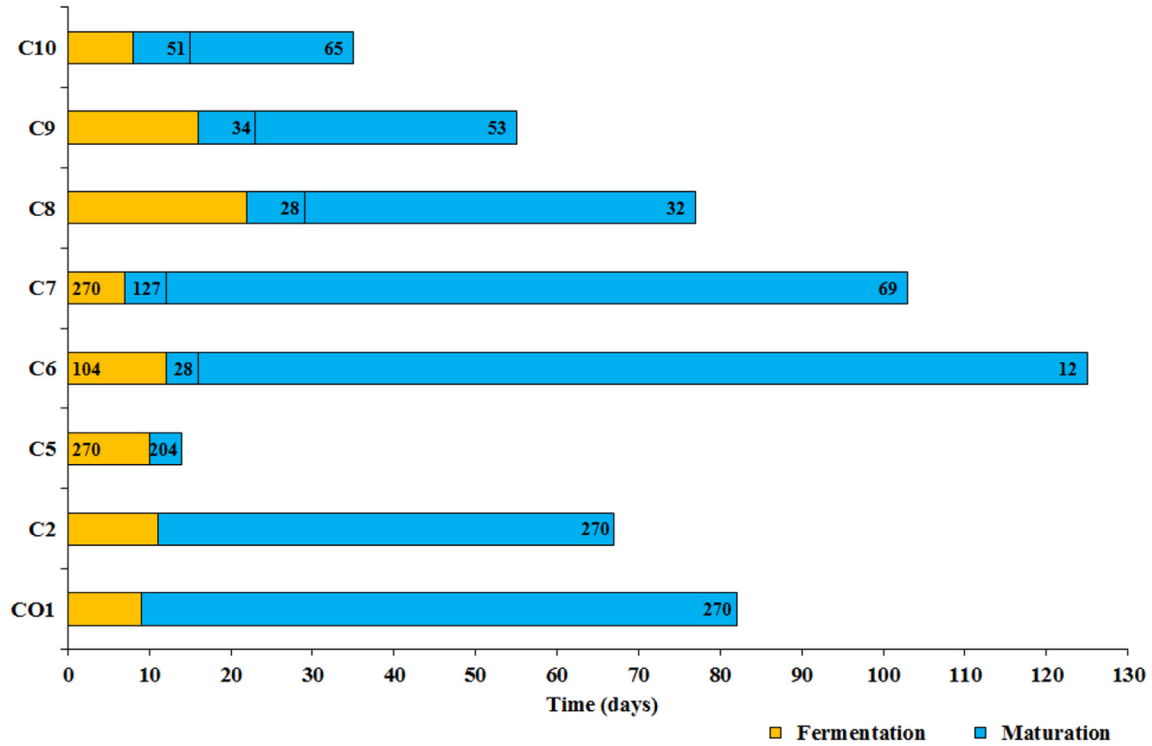

Fig. 3. Gluten concentration at different times after the brewing production processes. The value of 270 mg/L is the upper limit of measurable gluten concentration. Uncertainties not shown.

produced from tests CO1 and C2 were >270 ± 18 mg/L after 60–80 days since the beginning of fermentation.

The malts mix used in tests C5 and C6 were exactly the same, as well as the respective starch extraction efficiencies, in turn estimated based on measurements of sugar concentration in the wort during mashing, as explained in section 2.3, were nearly identical, *i.e.* 57% (C5) and 56% (C6). Moreover, the type and quantities of malts were very similar to tests CO1 and C2. Yeast strains and their respective quantities were identical, while the fermentation times were only slightly different (10 and 12 days, respectively). As shown in Fig. 2(c), the net process times before fermentation were different, resulting in electricity consumptions equal to 84 kWh and 108 kWh, respectively, i.e. much bigger than in tests CO1 and C2.

As shown in Fig. 3, test C6 showed a gluten concentration far lower than test C5 at any time: 104 ± 2.5 mg/L vs > 270 ± 18 mg/L at the beginning of fermentation, and 28 mg/L (*i.e.* just above the gluten-free threshold) *vs.* 204 mg/L approximately at the same time after test (14 and 16 days, respectively). Eventually, 125 days after brewing (excluding fermentation), beer produced by means of test C6 fell well below the gluten-free threshold, down at a mere 12 ± 0.3 mg/L concentration.

Few gluten concentration values during the brewing processes of tests C5 and C6 are shown in Fig. 4.

The differences in the initial temperature of each process before mashing-out (21 °C in test C5 and 57 °C in test C6) did not lead to any effect on gluten concentration at the time of mashing-out (both >270 ± 18 mg/L).

After restart following mashing-out, an additional hydraulic pressure was readily imposed in C6 process, oscillating between 0.5 atm and 2 atm and averaging at 1.5 atm, with approximately 30 kWh energy consumed during the overpressure stage, keeping the temperature at around 72 °C. As shown in Fig. 2(c), this resulted in far greater CN values for test C6, more than double the values at similar temperatures before the activation of the additional pressure, hinting to a more violent hydraulic cavitation regime. Rather surprisingly, Fig. 4 shows that the gluten concentration for test C6 fell far below the values observed for test C5 at the same energy consumption: always >270 ± 18 mg/L for test C5, while in test C6 it decreased to about 160 ± 11 mg/L and 104 ± 2.5 mg/L at the energy consumptions of 84 kWh and 108 kWh, respectively.

Test C7 involved the use of a marginally different dosage of malts, but with the same overall quantity as in tests C5 and C6, as shown in Table 1. Moreover, since the mashing efficiency was far greater in test C7 (71%) than in tests C5 and C6 (Albanese et al., 2017), one would expect that–all else being equal–the gluten concentration in the finished beer resulting from test C7 will be higher. No additional hydraulic pressure was ever applied, so that cavitation numbers were very close to those assessed during brewing in test C5. Actually, the gluten concentration in test C7 was always >270 ± 18 mg/L during brewing before fermentation, similar to test C5. Moreover, the fermentation time in test C7 was equal to 7 days, shorter than in both tests C5 (10 days) and C6 (12 days). One might therefore wonder why the gluten concentration in the beer from test C7, measured during maturation, was far lower than in test C5 just 5 days (C7) and 4 days (C5) after the end of fermentation, *i.e.* 127 ± 3 mg/L against 204 ± 14 mg/L, as shown in Fig. 2. Eventually, 103 days after brewing, beer produced by means of test C7 fell well below the very low gluten content threshold, at just 69 ± 2 mg/L.

As shown in Fig. 2(d), while the overall process time before fermentation was similar to tests C5 and C6, the far more efficient starch extraction resulted in mashing-out at an energy consumption equal to 27 kWh, against about 57 kWh for tests C5 and C6, translating into less than half time elapsed from process beginning. Therefore, a much larger fraction of the overall process time in test C7 occurs after mashing-out, *i.e.* with all the starch and its gluten content being available in the wort and undergoing the hydraulic cavitation processes.

The same gluten data for tests C5, C6 and C7 after yeast pitching, shown in Fig. 3, are reproduced in Fig. 5 in terms of the respective

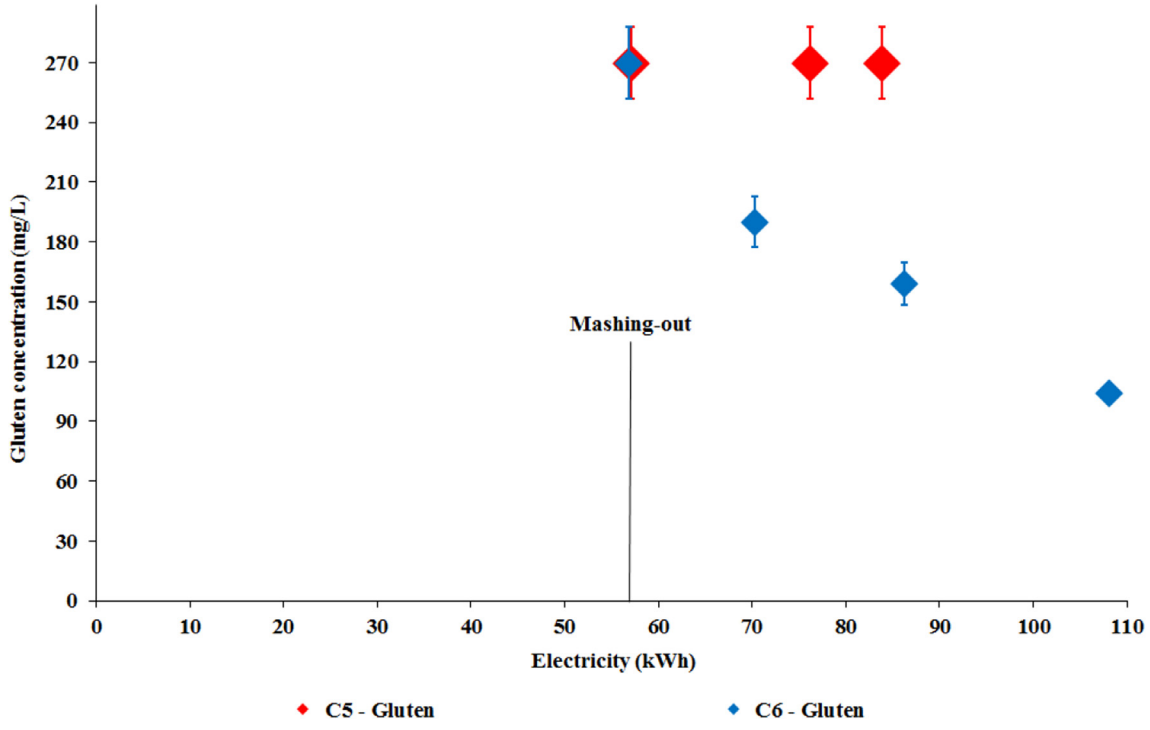

Fig. 4. Evolution of gluten concentration during brewing processes, after mashing-out, for tests C5 and C6.

absolute values as well as the percentage of initial values, with test C6 clearly outperforming all the others.

The three-data gluten concentration series concerning tests C6 and C7 are best fitted by a power decay function of time, with more than 99.5% of variance explained. If such relationship could be proved generally valid, it would provide predictive capability about the eventual achievement of either the very low gluten content (100 mg/L) or gluten-free (20 mg/L) thresholds, starting from given initial conditions, brewing recipe and operational HC parameters.

3.3. Effects of cavitation after yeast pitching

In tests C8 and C10, contrary to all the others, HC processes were activated also after yeasts pitching and, along with test C9, the subsequent fermentation occurred in the installation shown in Fig. 1. Moreover, Pale malt was used in place of Pilsner (Table 1).

Fig. 3 shows that the respective gluten concentrations were lower than the very low gluten content threshold, which is especially relevant for test C9 when no additional cavitation process was activated after yeasts pitching, which could be a result of using a different barley malt.

Recalling that those tests were carried out with exactly the same ingredients, Fig. 2(e) shows that the brewing processes up to mashing-out were practically identical too, as well as yeast pitching occurred at the same time in tests C8 and C9, in terms of energy consumption, and just earlier (4 kWh less consumed energy) in test C10. Up to yeast pitching, the consumed energy in those tests was far lower than in tests C5, C6 and C7. Moreover, all three tests were carried out at atmospheric pressure, producing the same cavitation numbers at the corresponding temperatures.

Immediately after yeast pitching, HC was activated in both tests C8 and C10, with energy consumption around 5 kWh and 19.5 kWh, respectively, while no HC was applied in test C9, in order to look for possible advantages brought by the cavitation processes at that stage, such as those claimed in a previous study (Safonova, Potapov, & Vagaytseva, 2015).

However, Fig. 6 hints to a definite relationship between fermentation time, *i.e.* residence time in the open vessel, and gluten concentration measured 7 days after bottling.

Regardless of any HC treatment after yeast pitching, the gluten concentration decreases with fermentation time according to a power decay function of time (>99.9% variance explained), despite its significance cannot be established based on only three data points. Such relationship seems to agree with the consideration expressed in Section 1 about the sensitivity of the proline assimilation rate to the availability of dissolved molecular oxygen, which increases with the residence time in an open vessel (Procopio et al., 2013). Therefore, no distinct effect of HC performed after yeast pitching can be inferred from the above-discussed data.

Nevertheless, Fig. 3 shown a rather surprising feature, *i.e.* the absence of any decay in gluten concentration during maturation: on the contrary, an increase with time was observed, largest in test C9 (from 34 ± 1 mg/L to 53 ± 1 mg/L during 32 days of maturation in bottles), and insignificant in test C8.

4. Discussion

4.1. Effects of cavitation before yeast pitching

Based on the results from tests CO1, C2, C5, C6 and C7, presented in Section 3.2 and concerning cavitation processes activated only before yeast pitching, enzymes do not look like to play any significant role in gluten reduction, since they are inactivated at the time of mashing-out, when gluten concentrations were at the top of the scale (>270 ± 18 mg/L).

After mashing-out and before yeast pitching, the decay of gluten concentrations observed in test C6, down to 104 ± 2.5 mg/L, which was completely absent in test C5 (Fig. 4), could be attributed to the thermo-mechanical shocks triggered under the respective violent cavitation regimes (CN > 0.3) during a sufficient time lapse, likely

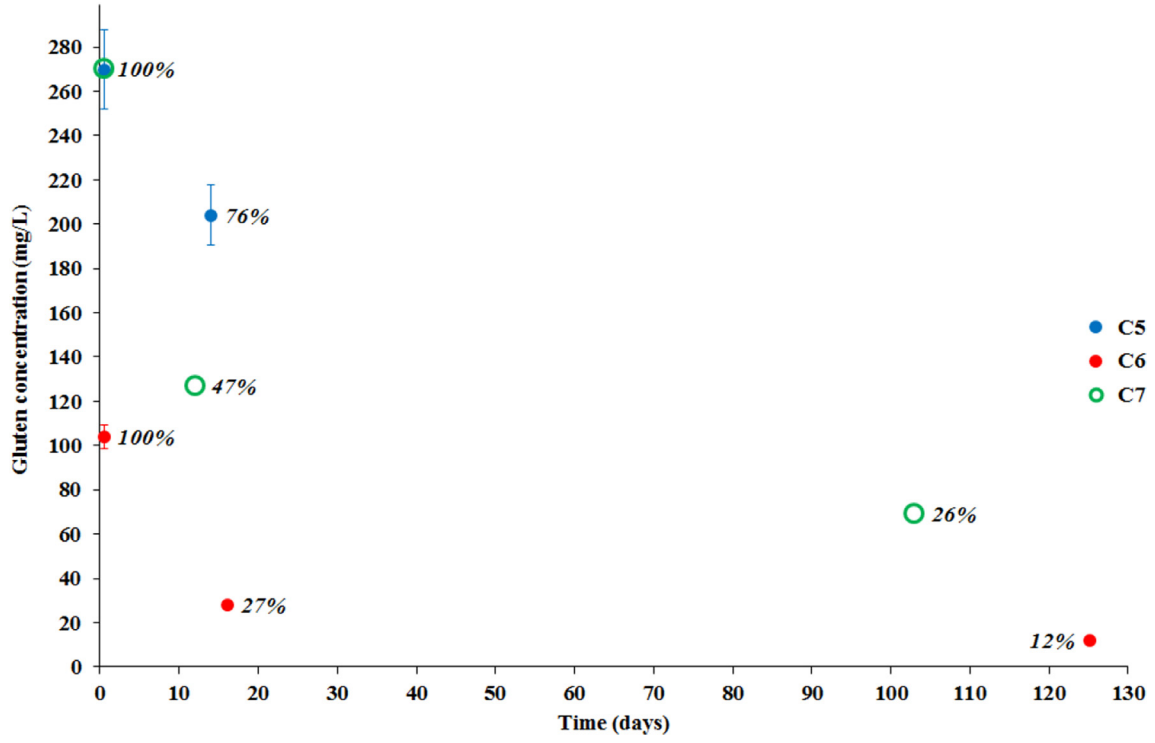

Fig. 5. Tests C5, C6 and C7: decay of gluten concentration as a function of time after the respective yeast pitching and start of fermentation.

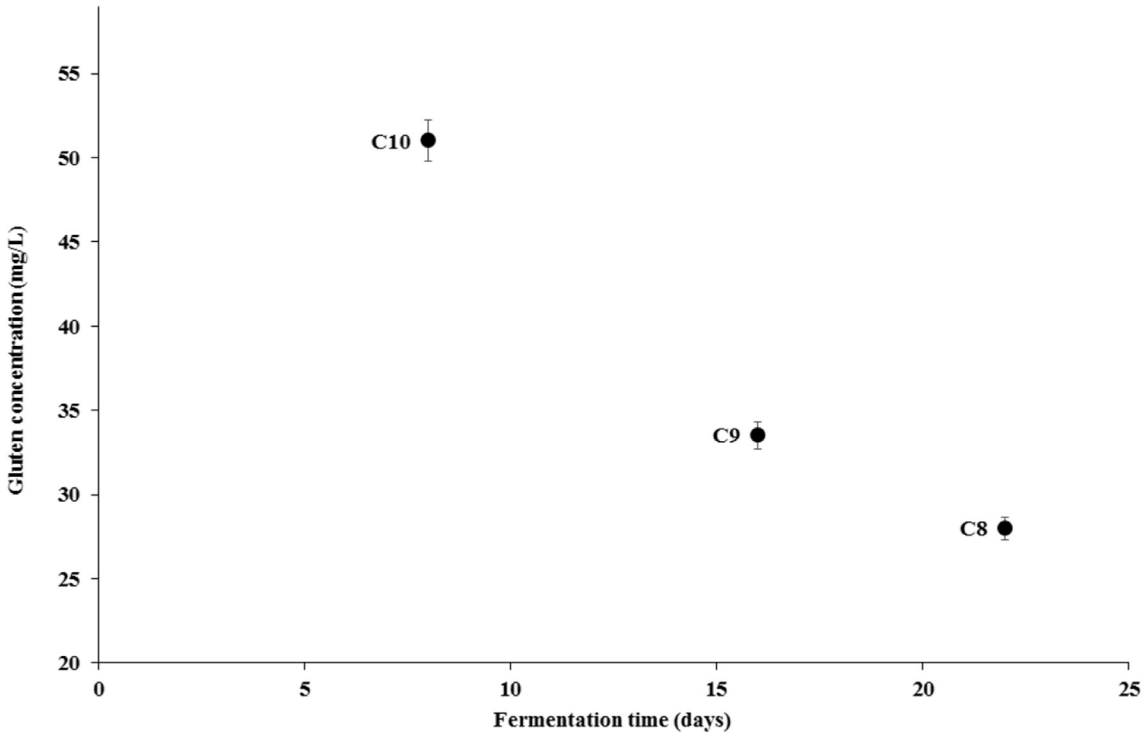

Fig. 6. Tests C8, C9 and C10: gluten concentration measured 7 days after bottling, as a function of the length of the fermentation period.

leading to a partial destruction and widespread degradation of proline residues.

Data shown in Fig. 2(c) for test C6 suggest an upper limit to the above-mentioned time lapse, sufficient to obtain gluten-free beer, in terms of specific consumed energy, of about 0.17 kWh/L, as resulting from about 30 kWh—consumed from mashing-out until CN > 0.1—divided by 170 L. While the identification of a lower limit to achieve gluten-free beer will require further tests, it is conceivable that such limit could be substantially lower, as Fig. 3 shows that the later gluten concentration in test C6, 125 days after brewing (excluding fermentation), was just 12 ± 0.3 mg/L.

A similar argument holds for test C7, carried out at atmospheric pressure, *i.e.* at a milder cavitation regime (CN < 0.2). Data in Fig. 2(d) suggest an upper limit to the HC process time after mashing-out, in terms of specific consumed energy and sufficient to obtain very low gluten content beer, of about 0.29 kWh/L (about 50 kWh consumed from mashing-out until CN > 0.1, divided by 170 L). Again, such limit could be substantially lower, as Fig. 3 shows that the later gluten concentration in test C7, 103 days after brewing (excluding fermentation), was just 69 ± 2 mg/L. However, a lower bound on such limit could be set by the corresponding data from test C5 (Fig. 2(c)), around 0.06 kWh/L (about 10 kWh divided by 170 L).

More in detail, a possible interpretation of the proline degradation/destruction mechanism under HC processes could start from the consideration that barley hordein proteins are rich in hydrophobic amino acids (Malalgoda & Simsek, 2016). Indeed, hydrophobic bonds contribute significantly to the stabilization of gluten structure, as well as such bonds are quite resistant to heat due to their strength increasing with temperature (Wieser, 2007).

The amino acid proline itself is strongly hydrophobic and is a major cause for the very low solubility of gluten in water (Rahaman, Vasiljevic, & Ramchandran, 2016; Widyarani, Sari, Ratnaningsih, Sanders, & Bruins, 2016). Moreover, like other hydrophobic amino acids, proline is mostly located in the interior of the protein in order to stabilize the protein itself in aqueous solution (Tanford, 1962). Therefore, in order to directly expose proline molecules to HC processes, first the containing proteins have to be unfolded.

Proline residues are contained in both soluble and insoluble protein fractions of gluten, namely gliadins and glutenins, respectively, the latter including high molecular weight subunits (Wieser, 2007). Only the soluble fraction—gliadins—undergoes progressive disorganization and unfolding as bulk temperature increases up to 100 °C, while unfolding events in glutenins reverse as temperature exceeds 80 °C (Stănciuc, Banu, Bolea, Patraşcu, & Aprodu, 2017).

The hypothesis is advanced here that intense and/or prolonged HC-induced pressure shockwaves and mechanical jets could be effective to unfold a fraction of at least the higher molecular weight glutenin (insoluble) subunits, acting synergically with heat at least up to temperatures around 80 °C.

Once gluten proteins have been partially unfolded and proline residues are directly exposed to HC processes, their hydrophobicity suggests a different mechanism for the respective degradation. In the presence of cavitation bubbles, hydrophobic molecules tend to locate at the water-vapor interface or inside the vapor bubbles, where they can be destroyed or degraded by thermal pyrolysis triggered by bubble's collapse, when transient temperatures on the order of 10,000 K can occur during time lapses as short as a microsecond (Bagal & Gogate, 2014; Gore, Kumar, Pinjari, Chavan, & Pandit, 2014; Rajoriya, Carpenter, Saharan, & Pandit, 2016).

Whatever the detailed mechanisms, the degradation of proline residues could be responsible for the respective subsequent enhanced assimilation by the yeast strains during fermentation and maturation, eventually leading to a gluten-free product.

However, few uncertainties remain. First, the upper bound to the scale of gluten concentration measurements at 270 mg/L could affect the assessment of the respective decay rates observed for tests C6 and C7. Second, and more important, the long-lasting mild cavitation occurring in tests C5 and C6 during starch extraction (*i.e.*, before mashing-out) could have somehow affected the subsequent

gluten concentration decay occurred already before yeast pitching in test C6, and during fermentation in test C5. Third, the sustained HC phases activated in tests C6 and C7 after the respective mashing-out points were performed mostly at temperatures of 72 ± 3 °C, while recent findings hint to the temperature of 60 °C to achieve the most aggressive cavitation in water—greatest bubble collapse rates, strongest temperature and pressure shocks (Dular, 2016)—possibly showing the way to further improvement in the destruction of proline residues.

Finally, full grain cavitation carried out in test CO1 showed no observable effect upon gluten concentration, suggesting that only wort cavitation intensity and duration after mashing-out, and possibly before, do affect gluten concentration. However, further focused tests with cavitating grains should be performed before definitively ruling out the respective role.

4.2. Effects of cavitation after yeast pitching

Based on the results from tests C8, C9 and C10, presented in Section 3.3 and concerning cavitation processes activated also after yeast pitching, first it should be recalled that yeasts did not undergo any cavitation process in test C9 and that gluten concentration did not drop during maturation, contrary to tests C6 and C7. The different behaviors could be ascribed to the above-discussed degradation of proline residues in tests C6 and C7.

In tests C8, C9 and C10, the regrowth of gluten concentration could be attributed to a possible FAN release during maturation from inactivated or dead yeast cells, along with the additional hypothesis that a significant fraction of such released FAN is non-degraded glutamine or—more likely—proline, *i.e.* gluten constituents. The hypothesis is supported by recent findings already discussed in Section 2.2 (Choi et al., 2015).

The larger increase of gluten concentration in test C9 in comparison with tests C8 and C10 could hint to a relevant role of some sort of yeast cells *activation* by HC processes in the two latter tests.

More in detail, Fig. 7 shows that, during the residence in the open vessel (fermentation stage), an inverse relationship seems to hold between the tendencies of the concentration of yeast cells and the FAN, particularly for tests C8 and C9, along with the far greater concentration — on average, almost double — of alive yeast cells in test C9, despite wide oscillations. Nevertheless, the FAN concentration curves from tests C8 and C9 link up towards the end of fermentation in test C9, about 15 days after yeast pitching.

Few tentative conclusions about the possible role of hydrodynamic cavitation after yeast pitching can be drawn.

First, yeast cells were partially inactivated by HC processes in tests C8 and C10, in agreement with previous work by the authors (Albanese et al., 2015), with no apparent increase of lethality produced by the almost four-times longer treatment applied in test C10. Second, in comparison with test C9, yeast cells in test C8, having been *activated* by the HC process after their pitching, were more efficient in proline assimilation and irreversible degradation via the yeast oxidase process (Procopio et al., 2013), the latter boosted by the longer fermentation time in the open vessel, so much that the simultaneously occurring effects of the reduction of alive yeast cells concentration and the activation of the survived ones compensate each other with respect to the impact on the FAN concentration. Such hypothesis agrees with the large difference in gluten concentration recovery during maturation shown in Fig. 3 for tests C8 and C9, as well as with the observation that fermentation in test C8 started at least an hour earlier than in any other test.

However, the mechanism underlying the alleged *activation* of the surviving yeasts remains an open issue, beyond general considerations about the selective survival of the most efficient cells and the increase of mass transfer between their cellular membranes and the surrounding wort containing the proline residues, both produced by hydrodynamic cavitation processes.

It should be noted as well that the safe preservation of the beer wort in an open vessel during long fermentation times, such as in

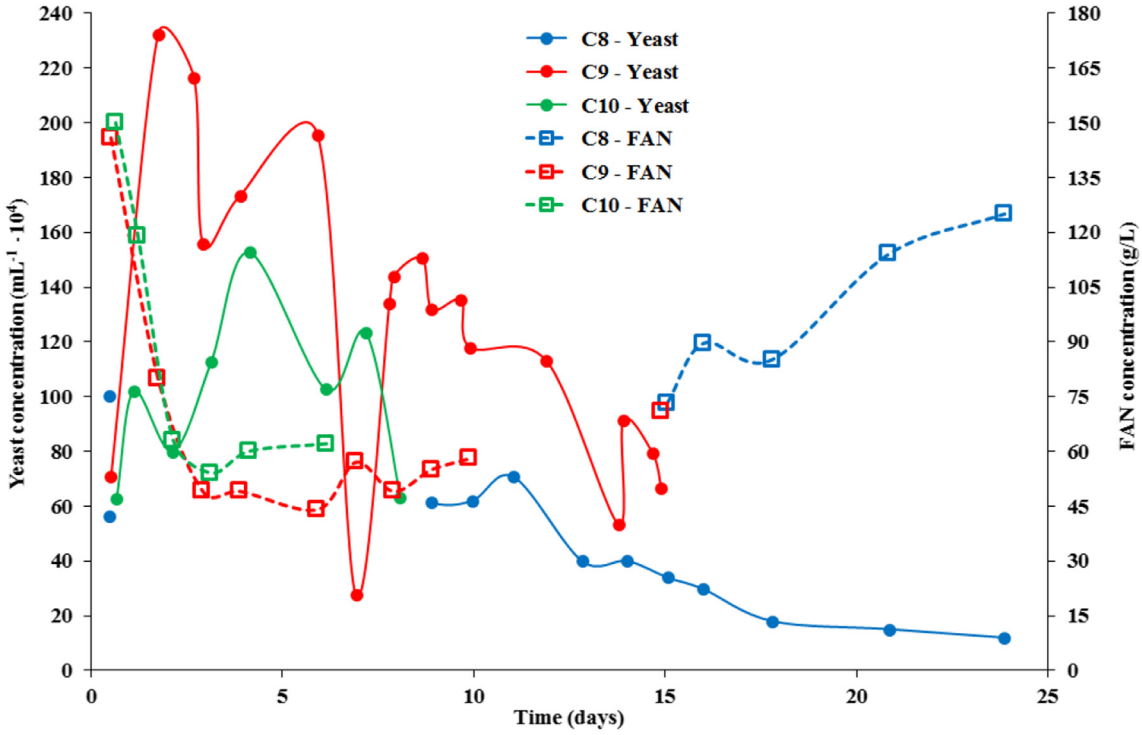

Fig. 7. Tests C8, C9 and C10: concentration of yeast cells and FAN during fermentation.

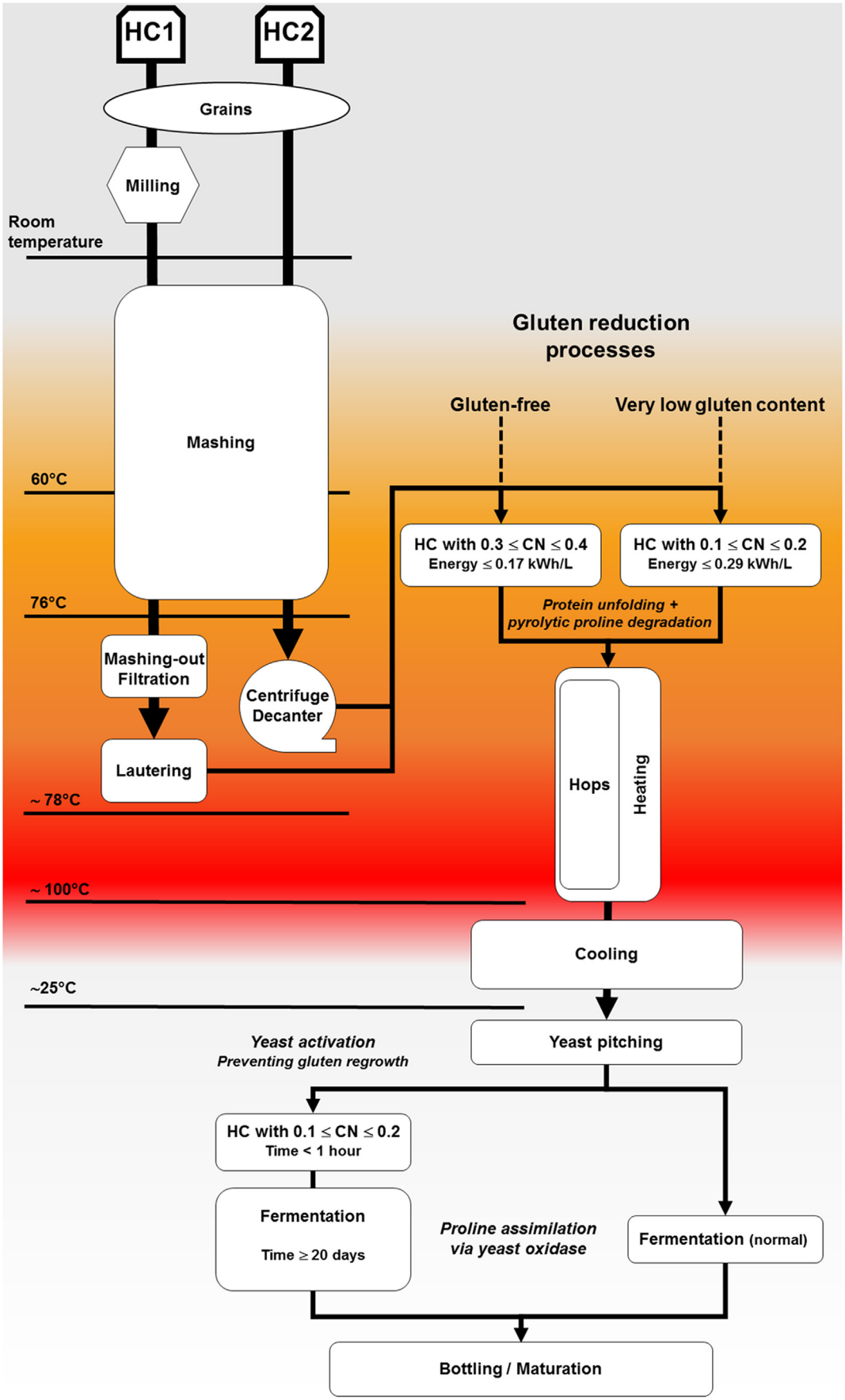

Fig. 8. Simplified scheme of the HC-assisted brewing processes leading to gluten concentration reduction in beers (HC1—BIAB setup, HC2—cavitating malts setup), along with the main hypothesized mechanisms of proline's degradation and assimilation.

test C8, could have been helped by the HC sterilization capabilities (Albanese et al., 2015, 2017). Moreover, it is remarkable that taste, aroma, flavor and foam stability of the finished beers from tests C8 and C10 have not been adversely affected by the HC treatment after yeast pitching, in agreement with what reported by a previous experience (Safonova et al., 2015).

In summary, what arises from tests C8, C9 and C10 is that the HC treatment of the beer wort after yeast pitching for the purpose of gluten concentration reduction should be short enough (*e.g.* < 1 h) to avoid inactivation of a too large fraction of the yeast cells, as well as coupled to longer than normal fermentation time (*e.g.* more than 20 days).

4.3. Overall processes leading to gluten reduction

Fig. 8 shows a simplified scheme of the processes leading to gluten concentration reduction in wort and beer by means of HC-assisted brewing, along with the main above-advanced hypotheses about the mechanisms of proline's degradation and assimilation.

Limited to the brewing recipes, *i.e.* to the specific mix of barley malts, considered in the described experiments, after mashing-out, the wort may be subjected to a relatively short, violent cavitation process ($0.3 \leq CN \leq 0.4$), or to a longer, mild cavitation process ($0.1 \leq CN \leq 0.2$), to be carried out at temperatures in the range 60 °C–70 °C, eventually leading to gluten-free or very low gluten content beers, respectively.

After yeast pitching, just before the beginning of the fermentation stage, possible later regrowth of gluten concentration in the wort and beer can be prevented by means of a short-term, mild cavitation process ($0.1 \leq CN \leq 0.2$), to be carried out at temperatures consistent with the fermentation stage (usually lower than 25 °C).

5. Conclusions

The new controlled hydrocavitation-assisted beer brewing technique, developed by the authors (Albanese et al., 2017), provides another important advantage over conventional brewing technology, achieving greatly reduced gluten concentration in the resulting beers. Eventually, in correspondence of suitable cavitation regimes identified in this study for barley malts, the gluten concentration found in the beer is lower than the "gluten-free" threshold (20 mg/L) or the very low gluten content threshold (100 mg/L). This new route to gluten reduction can be important because it allows retaining the same ingredients and recipes of standard beers, while avoiding any chemical additives or proprietary techniques (such as filtration, ultrafiltration, enzymatic compounds and silica gel), preserving taste, flavor and aroma of the best craft beers. As well, no drawbacks arose, such as oxidation of wort or beer, and damage to the equipment.

Hydrodynamic cavitation was shown to be quite effective during mashing and possibly at the beginning of fermentation, *i.e.* after yeast pitching, with early operational guidance provided for both brewing stages. Preliminarily, we ascribe this newly observed phenomenon to the partial destruction and degradation of proline residues, by means of suitable HC processes, as well as hypotheses are advanced about the pathways to proline degradation.

General recommendations for future research follow the ones already issued in the authors previous study (Albanese et al., 2017), *i.e.* carrying out further experiments, both as duplicates of the herein discussed ones and more focused in order to further restrict the range of operational parameters, using different cavitation reactors such as slit Venturi, and assessing the quality of the very low gluten content or gluten-free beers by means of independent panels. More specific recommendations concern the direct observation of the evolution of proline residues at the molecular level, as well as the identification of the operational parameters and ranges in correspondence with different brewing recipes.

Declaration of interest

L.A. and F.M. were appointed as Inventors in the patent submitted on August 9, 2016, international application No. PCT/IT/2016/000194 "A method and relative apparatus for the production of beer", pending.

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