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The impact of the physiological condition of the pitching yeast on beer flavour stability: an industrial approach

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Abstract

Studies on the investigation of the impact of the physiological condition of the pitching yeast on beer flavour stability are reported. Fermentation trials were conducted at industrial scale using a commercial lager brewing yeast at different physiological states. Fermentation performance was followed by monitoring yeast growth, ethanol synthesis, original gravity and attenuation throughout the fermentative process. Beer flavour was evaluated through the content of higher alcohols and volatile esters. After an extended storage, beers were evaluated by an expert sensory panel and the concentration of 2-furaldehyde and E-2-nonenal was assessed.

The results demonstrate that the parameters under study are little affected by the physiological condition of the pitched yeast, except for the cell growth which is higher for the yeast pitched with lower vitality. Additionally, a recovery of the yeast vitality is observed at the end of fermentation. A marked improvement on the beer flavour stability can be achieved by increasing vitality of the pitching yeast, according to the statistically significant (t-test, $p < 0.1$) differences observed in the sensory evaluation.
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Keywords: Beer; Flavour stability; Yeast physiological condition; Vitality; Fermentation performance

1. Introduction

Flavour stability, an important quality criterion for beer, has become one of the most important topics in brewing research over the past few years. High-temperature storage to which the beer is occasionally exposed results in accelerated staling and is often accompanied by the development of an oxidized odour/flavour (papery, cardboard flavour) (Madigan, Perez, & Clements, 1998). Breweries have directed their efforts to producing beers with the highest possible organoleptic stability, in order to meet consumer's requirements of drinking a beer with an ideal taste during the shelf life indicated.

In the brewing process, the efficiency of fermentation and the character and quality of the final product are intimately linked with the amount and health of the yeast being pitched. The physiological condition of the pitching yeast may influence the levels of organic acids, esters, higher alcohols, aldehydes and diacetyl throughout fermentation and maturation, and consequently contribute to the overall organoleptic properties of the final beer (Heggart et al., 2000). Predicting the capacity of pitching yeast towards its subsequent fermentation performance is therefore a requisite. The propensity to perform or yeast quality is usually described in terms of its ''viability'' or its ''vitality'' (Lentini, 1993). Yeast viability is an expression used to describe the ability of cells to grow, reproduce and interact with their immediate environment (Smart, Chambers, Lambert, & Jenkins, 1999). Yeast vitality has been variously described as a measure of activity (D'Amore, 1992), fermentation performance (Boulton, 1991), or the capacity to overcome and recover from physiological stress (Smart, 1996).

Methods for assessment of viability and vitality of brewer's yeasts were reviewed elsewhere (Heggart et al., 2000). The evaluation of yeast viability includes methods

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based on cell replication, methods using vital stains, and methods based on measurement of cellular products resulting from metabolic activity (Jones, 1987). Yeast vitality methods usually involve the measurement of specific yeast cell components critical to fermentation activity, or the determination of a metabolic activity that may be related to fermentation performance (Axcell & O'Connor-Cox, 1996).

The fermentation performance of yeast is also influenced by external factors, like wort clarity, lipid composition, wort oxygenation, pitching-rate and temperature, among others. Although few works have been published regarding the direct impact of yeast physiological state on the flavour stability of finished beer, it is already known that the ability of yeast to produce sulfur dioxide and its ability to reduce carbonyl compounds are the main factors involved. In this work an industrial application in order to investigate the potential role of the physiological condition of a lager pitching yeast on the beer flavour stability is described.

2. Experimental

2.1. Yeast

A lager brewing strain of *Saccharomyces cerevisiae* var. *uvarum* (Unicer Strain II) was used throughout. Lager fermentation trials were conducted in duplicate at industrial scale in 1000-HL cylindroconical fermenters.

2.2. Wort

A lager wort (14 °P) brewed according to standard production procedures was used throughout. The wort (pH between 5.1 and 5.3) was filtered in the *Meura 2001* filter system and then cooled. In order to preserve the mashing-in and brewing conditions as similar as possible, the same brew was equally divided for fermentations 1 and 2 and for the fermentations 3 and 4, respectively. Approximately 1000 HL of the wort was transferred into each of the four cylindroconical fermenters and aerated in-line (8 mg/L of dissolved oxygen).

2.3. Fermentation conditions

Fermentations were undertaken using a bottom fermenting yeast. Four fermentations were conducted under the conditions as similar as possible, except for the quality of the pitching yeast. The same wort, resulting from the same brew, was equally transferred into two cylindroconical fermenters (fermentations 1 and 2). A second wort, produced in a different brew, was poured into two fermenters, identified as 3 and 4. For fermentations 1 and 3 the higher vital pitching yeast was used, whereas the wort for fermentations 2 and 4 was pitched with the lower vital yeast. The physiological condition of the yeast was evaluated by measuring both viability and vitality, as described below. The fermentation conditions are outlined in Table 1.

2.4. Fermentation analysis

Yeast cell counts were performed using a Model ZM Coulter counter (Schisler, Ruocco, & Mabee, 1982). Beer gravity (°P), fermentability (% real degree of fermentation) and ethanol were monitored using a SCABA 5600 Automatic Beer Analyser (Tecator AB, Sweden) (American Society of Brewing Chemists, *Methods of Analysis*, 8th rev. ed., St. Paul, MN, 1992).

Total diacetyl was measured throughout fermentation and maturation by gas chromatographic analysis of the static headspace as recommended by the European Brewery Convention (*Analytica EBC*, Nürnberg: Fachverlag Hans Carl, 2000).

2.5. Viability measurement

In all cases, the viability was measured using the methylene blue staining technique (Pierce, 1970). The method is based on the generally accepted fact that methylene blue is an autoxidizable dye that, upon entry into the cytoplasm of living cells, is oxidized to its colourless leuco-form. Inability of a cell to stain is an indication that the rate of influx of the dye through the membrane is balanced with the rate of oxidation of the stain (Heggart et al., 2000).

Table 1
Fermentation operating parameters

Parameter[a]	Fermentation 1	Fermentation 2	Fermentation 3	Fermentation 4
Wort OG (°P)	14.4	14.5	14.3	14.4
Initial temperature (°C)	8.9	8.9	8.9	8.9
Initial DO (mg/L)	8.0	8.0	8.0	8.0
Vitality of the pitching yeast (AU)	8.6	3.5	8.6	3.5
Viability of the pitching yeast (%)	92	83	92	83
Fermentation cycles of the pitching yeast	1	5	1	5
Pitching rate (mL^{-1})	2.1×10^7	1.8×10^7	1.9×10^7	1.7×10^7

[a] OG: original gravity; AU: arbitrary units; DO: dissolved oxygen, measured prior to pitching using an Orbisphere meter (model 26131).

2.6. Vitality measurement

At present, it is generally accepted that no one technique accurately predicts yeast vitality under various brewing condition. In this work, vitality of the yeast was assessed on the basis of the "vitaltitration" method (Rodrigues et al., 2004), which was shown to correlate well with the usually used method, the acidification power test. The method measures the ability of the yeast to overcome, or better adapt to, an hostile environment, such as the increase of the medium pH. Vitality is expressed by VT (μmol NaHO/min) which is obtained by the following equation: [(conc. of NaHO) × (vol. NaHO used) × 10^6]/(pH drop time).

2.7. SO₂ and acetaldehyde analysis

Total SO_2 was measured at the end of fermentation and in the finished beer using the *p*-rosaniline method, as recommended by the American Society of Brewing Chemists (*Methods of Analysis*, 8th rev. ed., St. Paul, Minnesota, 1992). Acetaldehyde was determined by the method of Delcour, Caers, Dondeyne, Delvaux, and Robberechts (1982).

2.8. Esters and higher alcohols analysis

Ethyl acetate, isoamyl acetate, isopropyl, isobutyl and isoamyl alcohol were determined in finished beer using a Varian Star 3400 gas chromatograph equipped with a flame ionization detector (FID) and a CombiPAL Autosampler (CTC Analytics) as recommended by the Institute of Brewing (*Recommended Methods of Analysis*, London, 1997).

2.9. 2-Furaldehyde analysis

2-Furaldehyde analysis was conducted using an HPLC system incorporating a 307 piston pump and a 115 UV detector (Gilson) connected to the SP4600 integrator (Spectra-physics). The column used was a 25 cm × 4.6 mm i.d. Nucleosil C_{18} 5 μm (Phase Sep). 50 mL of carbonated beer was subjected to steam distillation until 50 mL of distillate was collected. Aliquots (20 μL) of samples were injected onto the HPLC system described above. The mobile phase was 50% (v/v) acetonitrile in water, supplied at 1.0 mL/min. Detection was by ultraviolet absorbance at 280 nm.

2.10. E-2-Nonenal analysis

The method for the extraction and quantification of *E*-2-nonenal was developed in this laboratory and is described elsewhere (Santos et al., 2003).

2.11. Sensory evaluation

Eight tasters of the internal sensory panel of UNICER (the main Portuguese Brewery Group) with at least one year of sensory experience were recruited based on their good sensory ability to identify the stale off-flavours. The panel was contracted for three 1 h sessions per week, which took place on the morning at an adequately isolated taste room. Panellists were asked to comment on the general quality (discrimination test) as well as to describe the flavour profile according to a special form (description test). The beers were tasted in random order, after natural aging (12 weeks at 20 °C) and forced aging (1 week at 37 °C) by the Duo–Trio test in which the same beer stored at 4 °C was used as the reference. All beers were tasted at 4 °C and evaluated for the degree of staling on a five-point scale defined as: +1, no sign of oxidation; 0, very slight oxidation symptoms; −1, slight oxidation symptoms; −2, enough level of oxidation to reject the beer and −3, very strong level of deterioration by oxidation. A comparison of the mean experimental values obtained for the total taste score was applied by a *t*-test, in order to evaluate the statistically different beers at 90% probability.

3. Results and discussion

3.1. Effect of yeast physiological condition on the fermentation performance factors

Several factors are known to affect the yeast fermentative capacity. Of particular importance in this respect is the composition of the medium. The pitching wort was checked for the free amino nitrogen (FAN) content by the ninhydrin method according to the recommended methods of the Institute of Brewing (*Recommended Methods of Analysis*, London, 1997), as well as for fermentable sugars, glucose, fructose, maltose and maltotriose (Method Wort-14, American Society of Brewing Chemists, *Methods of Analysis*, 8th rev. ed., St. Paul, MN, 1992). The carbohydrate profile was found to be similar for all the pitching worts (data not shown). The pitching rate is important in terms of the subsequent yeast growth and synthesis of metabolic by-products, thus the pitching rate was practically similar for all the trials (Table 1). Fermentative capacity, calculated from the decrease of specific gravity, caused by the decomposition of wort sugars by yeast, is more evident for the trials 1 and 3 during the first 1.5 days of fermentation (Table 2). During this period, the increase in yeast cells was in fact negligible (yeast count at Table 2) showing that the fermentative capacity is not due to the yeast growth or multiplication, but instead to the intense metabolic activity, which is well correlated with the vitality of the pitched yeast (Table 1). The higher

Table 2
Fermentation parameters monitored throughout the lager fermentation trials

	Fermentation[a]	Days of fermentation					
		0	0.5	1.5	2.4	4.4	6.0
Gravity (°P)	1	14.4	13.8	12.7	11.1	8.4	5.8
	2	14.5	14.1	13.3	11.7	8.6	5.6
	3	14.3	13.8	12.7	11.4	8.4	5.0
	4	14.4	13.8	13.2	12.3	8.8	6.1
Ethanol (% v/v)	1	–	0.4	1.0	1.8	3.4	4.8
	2	–	0.2	0.6	1.5	3.2	5.0
	3	–	0.3	1.0	1.6	3.3	5.1
	4	–	0.2	0.6	1.3	3.1	4.5
Yeast count (millions/mL)	1	21	–	23	37	39	22
	2	18	–	19	40	43	40
	3	19	–	20	40	39	27
	4	17	–	18	45	49	41
RDF[b] (%)	1	–	3.8	10.3	19.7	35.8	55.3
	2	–	2.1	6.6	15.9	34.5	56.2
	3	–	3.3	10.3	17.7	35.3	55.2
	4	–	2.0	6.8	11.8	29.6	48.2

[a] Fermentations 1 and 3: higher vital pitching yeast; fermentations 2 and 4: lower vital pitching yeast.
[b] RDF : real degree of fermentation.

ethanol yields obtained after 1.5 days for fermentations 1 and 3 are also indicative of higher yeast activity and thus of higher fermentation rates. Yeasts 1 and 3 fermented more rapidly early in the fermentations, confirming that the yeast vitality may play an important role during the first hours of fermentation. Final specific gravity as well as final ethanol yield (Table 2) did not correlate to early fermentation rates. The results obtained for the real degree of fermentation (Table 2) are consistent with those previously discussed, showing that the yeast propagated with higher vitality performs well in fermentation. The profile of fermentation 4 indicates a lower final ethanol concentration and a lower fermentability (Table 2). The fact that the yeast growth profiles show a lower multiplication for fermentations 1 and 3 after 4.4 days of fermentation (Table 2) suggests that an higher fermentative capacity may prevent an active yeast growth and multiplication. Van Hoek, Van Dijken, and Pronk (1998) observed a negligible increase in biomass concentration and an increase in ethanol concentration for the *S. cerevisiae* strain DS28911 showing the highest fermentative capacity. Nilsson, Larsson, and Gustaffson (1995) examined the catabolic capacity of *S. cerevisiae* in relation to its physiological state and maintenance requirement. Interestingly, stationary phase cells showed a lower respiratory capacity but almost as high fermentative capacity as cells originating from the logarithmic phase of growth. Our results are also in agreement with the concept of growth potential and fermentative capacity not being closely related. By selecting an yeast in a good physiological state, a rapid fermentation without excessive yeast growth may be achieved, in order to produce a beer with

the maximum attainable ethanol content, consistent with the overall flavour balance of the product.

Harvested yeast, in the form of yeast slurry, was assessed for viability and vitality at the end of fermentation. Fig. 1 plots the results for the pitching yeast and for harvested yeast after eight days fermentation. It is interesting to note that the viabilities were greatly increased during fermentation, although remaining lower for yeast slurry samples 2 and 4 (Fig. 1(b)). On the other hand, a substantial increase of the vitality was observed for the yeast slurry samples 2 and 4. It can be seen that at the end of fermentation, no significant differences were observed for the physiological state of yeast cropped from each of the four studied fermentations (Fig. 1(a)). This observation shows that yeast can recover its vitality throughout fermentation although this fact may not predict the capacity for yeast to overcome forthcoming stress situations such as storage, acid washing or other yeast management procedures. From these results it appears that during the active yeast growth the cells were able to trigger their stress resistance mechanisms.

3.2. Effect of yeast physiological condition on the concentration of beer volatile esters and higher alcohols

A recently published work indicated that exogenous diacetyl removal rates were influenced by yeast physiological condition and that the efficacy of diacetyl removal from an in vitro assay medium was enhanced where yeast had competent membranes (Boulton, Box, Quain, & Molzahan, 2001). In this investigation, diacetyl content was monitored over fermentation (data not
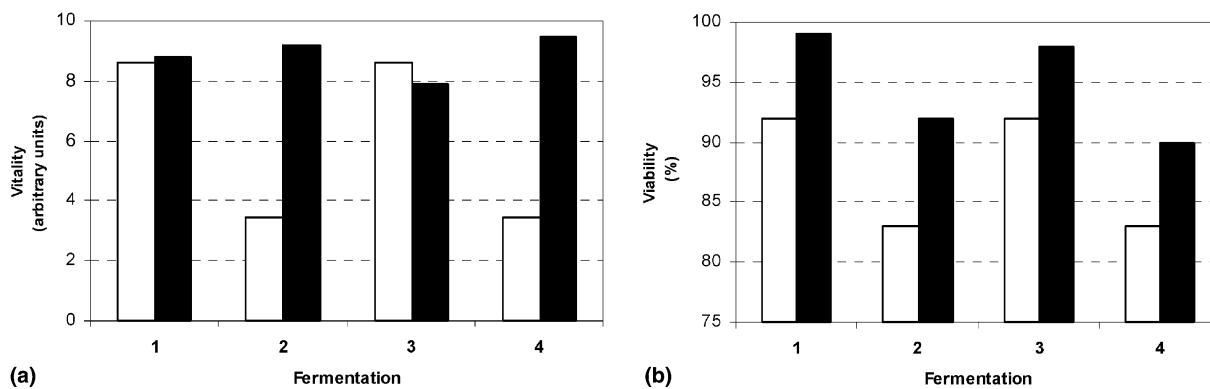
Fig. 1. (a) Vitality and (b) viability of pitching yeast (□) and of harvested yeast after eight days of fermentation (■) for the lager fermentation trials. Fermentations 1 and 3: higher vital pitching yeast; fermentations 2 and 4: lower vital pitching yeast.

shown) but no correlation was found between the yeast physiological condition and the ability to reduce diacetyl. During brewing fermentation the rate limiting factor for diacetyl removal is the α-acetolactate oxidative decarboxilation, which may explain this observation. Higher alcohols and volatile esters are metabolic by-products contributing to the regulation of intermediary metabolism in yeast during fermentation (Quain, 1988). The increased yeast growth for the fermentations 2 and 4 can explain the higher concentration of higher alcohols (fusel oils) observed in finished beer although practically no difference was noticed for esters (Table 3). Several factors are known to affect the yeast metabolism, including the wort composition, initial wort aeration, the propagation temperature and the pitching rate. We believe that the lower levels found for higher alcohols in the beers fermented by the higher vital pitching yeast is caused by the intense initial fermentative activity, thus producing less metabolic by-products of amino acid synthesis. The same fermentation conditions may influence the sulfur dioxide and acetaldehyde levels (Fig. 2). As sulfite production is more active when yeast growth slows down or stops, all conditions which promote yeast growth will reduce the amount of sulfite excreted in the medium (Dufour, 1991). Accordingly, fermentations 1 and 3 which show the lower yeast cells count (Table 2) secreted higher levels of sulfite (Fig. 2). In the present experiments yeast in a better physiological
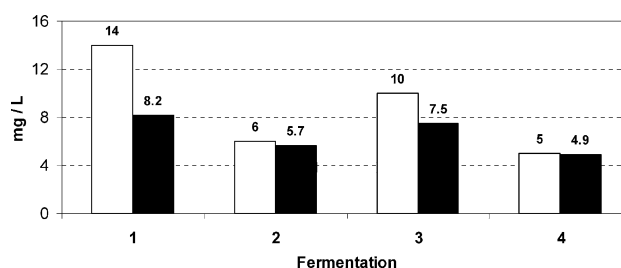

Fig. 2. The effect of yeast physiological condition on total SO_2 (□) and acetaldehyde (■) contents at the end of fermentation. Fermentations 1 and 3: higher vital pitching yeast; fermentations 2 and 4: lower vital pitching yeast.

condition (fermentation trials 1 and 3), which fermented more rapidly early in the fermentation, showed slower growth and lower multiplication and also secreted lower levels of fusel alcohols and higher levels of sulfite than the yeast in the worse physiological condition (fermentation trials 2 and 4).

Fermentation conditions that enhance yeast sulfite production and secretion will allow the formation of sulfite-carbonyl adducts from carbonyls present in the wort and prevent their further metabolism by the yeast (Dufour, 1991). In this manner carbonyls such as acetaldehyde and diacetyl may be transferred to the beer. The high amount of acetaldehyde found for fermentations 1 and 3 (Fig. 2) may be explained by the formation of adducts with sulfite, rendering unfeasible the reduction to ethanol. The ability of sulfite to prevent the appearance of the carbonyl compound *E*-2-nonenal during beer ageing has been demonstrated by brewing beer with a yeast strain blocked in the sulfur assimilation pathway (Johannesen, 1999).

3.3. Effect of yeast physiological condition on the beer flavour stability

Among the many aromatic volatiles which contribute to the flavour of beer, the higher unsaturated aldehydes with a 6–12 carbon chain have particularly low

Table 3
The effect of yeast physiological condition on beer higher alcohols and volatile esters contents (mg/L)

	Beer 1	Beer 2	Beer 3	Beer 4
Isopropanol	17.7	17.1	18.8	17.4
Isobutanol	11.6	13.3	11.9	13.2
Amyl alcohol	68.9	72.8	68.8	74.4
Ethyl acetate	17.0	17.3	17.1	16.7
Isoamyl acetate	1.20	1.21	1.14	1.17
Ratio alcohols/esters	5.4	5.6	5.4	5.9

Beers 1 and 3: higher vital pitching yeast; beers 2 and 4: lower vital pitching yeast.

organoleptic thresholds (Meilgaard, 1975). *E*-2-nonenal, which is member of this group has an extremely low flavour threshold of 0.1 ppb and contributes an unpleasant straw-like, "cardboard" flavour to the beer. It was decided to monitor *E*-2-nonenal and 2-furaldehyde, two carbonyl compounds well known to be good indicators of beer aging, during an extended storage at room temperature as well as after a forced aging test. The concentration of *E*-2-nonenal was assessed for the pitching wort and also after 12 and 36 h of fermentation (Fig. 3(a)). During fermentation the yeast oxido-reductases reduce aldehydes to the corresponding alcohols which have much higher taste thresholds. The concentration of free *E*-2-nonenal is thus much higher in pitching wort (between 0.64 and 0.71 µg/L) than in beer. The reduction of *E*-2-nonenal seems to be rapid and after 36 h of fermentation time the concentration of this compound is evenly reduced by both yeasts, although it seems that the lowest vital yeast is more efficient. Accordingly, the amount of *E*-2-nonenal reduced in the first 36 h did not reflect the yeast fermentation performance and was not correlated with pitching yeast vitality. Naturally (12 weeks at 20 °C) and forced (7 days at 37 °C) aged beers were analyzed for the content of *E*-2-nonenal (Fig. 3(b)). Beer 1 shows the highest amount of this aldehyde, which is in accordance to the *E*-2-nonenal level found in the pitching wort (Fig. 3(a)). These results suggest that the large amount of *E*-2-nonenal (and precursors) released during mashing and wort boiling may therefore play an essential role on the beer flavour stability. The formation of adducts between
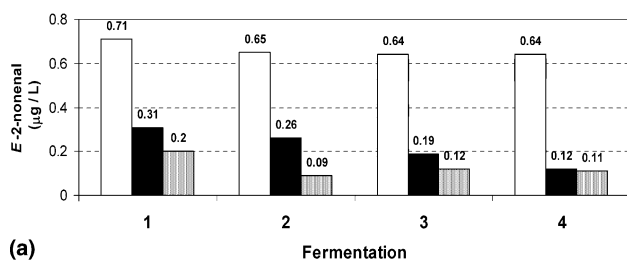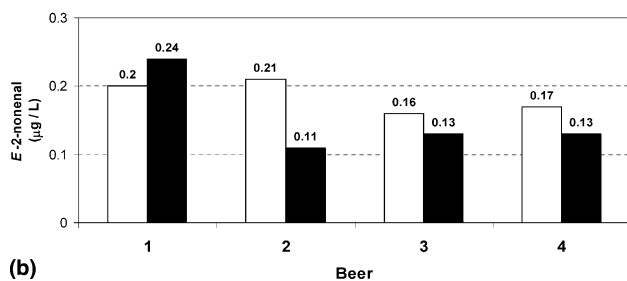
sulfite and *E*-2-nonenal during the final phase of fermentation 1, which produced 14 mg/L of SO_2 (Fig. 2), may explain the higher levels of *E*-2-nonenal found for fermenting wort 1 and for the corresponding beer. Investigation of the significance of these adducts for enzymatic reduction of carbonyl compounds is important to understand the mechanism of beer flavour instability.

5-Hydroxymethylfurfural and 2-furaldehyde are chemical indices of heat damage, formed by the Maillard reactions between sugars and aminoacids during malting and wort boiling (Madigan et al., 1998). 2-Furaldeyde can therefore be used as a good analytical marker of heat damage of beer. Fig. 4 depicts the 2-furaldehyde content for the fresh and aged beers. A clear increase can be seen for the aged beers but no significant differences were observed between the fermentation trials, although the 2-furaldehyde content for the fresh beer is slightly higher in the case of fermentations 2 and 4.

Well-trained panel members followed the degree of staling of each group of beers during an extended storage period at room temperature, as well as after forced aging (Fig. 5). The sensory data was examined to assess if they were significantly different at a chosen level of probability by a *t*-test. The results were surprisingly in disagreement with those obtained for the chemical indicators. Although *E*-2-nonenal and 2-furaldehyde
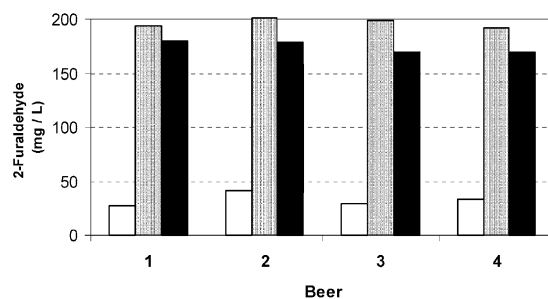
Fig. 4. The effect of yeast physiological condition on the 2-furaldehyde concentration for fresh beer (□), forced aged beer (▨) and naturally aged beer (■). Beers 1 and 3: higher vital pitching yeast; beers 2 and 4: lower vital pitching yeast.

(a)

(b)

Fig. 3. Effect of the yeast physiological condition on the *E*-2-nonenal content throughout the lager fermentation trials (a) and for the aged beers (b). (a) □, pitching wort; ■, 12 h of fermentation; ▨, 36 h of fermentation. (b) □, forced aged beer; ■, naturally aged beer. Fermentations (beers) 1 and 3: higher vital pitching yeast; fermentations (beers) 2 and 4: lower vital pitching yeast.
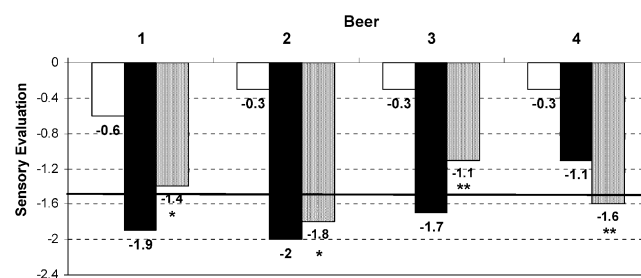
Fig. 5. Sensory evaluation of fresh (□), forced (■) and naturally (▨) aged beers. Beers 1 and 3: higher vital pitching yeast; beers 2 and 4: lower vital pitching yeast. The filled line represents the threshold of staling flavour (−1.5 in the sensory scale). Beers marked with one asterisk (*) and two asterisks (**) are significantly different (based on a *t*-test, $p < 0.1$).

content for aged beers did not reflect the yeast fermentation performance, sensory analyses prove to be significantly correlated (*t*-test, $p < 0.1$) to the pitching yeast vitality. After 12 weeks of storage at 20 °C the beers produced with the highest vital pitching yeast (beers 1 and 3) were satisfactorily evaluated (average −1.3) whereas beers 2 and 4 were considered not satisfactory (average −1.7) by the sensory panel. A statistically significant improvement (*t*-test, $p < 0.1$) of the beer flavour stability could be achieved in these industrial assays by choosing a pitching yeast in a better physiological state, as can be confirmed by comparing in between the degree of staling for the beers marked with one asterisk (*) and beers marked with two asterisks (**). It is important to recall that fermentations 1 and 2 use wort of the same brew and the same applies to fermentations 3 and 4. The forced aged test seems to be extremely hostile and do not reflect the natural sensory changes during beer staling, as confirmed by the differences in 2-furaldehyde concentration (Fig. 4). The differences observed in the sensory evaluation of naturally aged beers, established at the 90% confidence level, are even more evident considering that the filled line (−1.5 in the sensory scale) depicted in the Fig. 5 represents the frontier between satisfactory/not satisfactory beers. These results suggest the importance of controlling the physiological stability of the pitching yeast in order to improve the organoleptic properties of the final beer.

Even though the results presented for the fermentation trials did not enable us to gather essential chemical data, there are now evidences at industrial scale on the importance of the yeast physiological condition for the production of higher quality beers. The influence of the yeast vitality on the overall organoleptic properties of the final beer is now recognized by the Portuguese brewery companies, as demonstrated by the inclusion of the "vitaltitration" method as a routine assay for the yeast quality assessment.

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