Effects of Lysozyme on the Microbiological Stability and Organoleptic Properties of Unpasteurized Beer

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ABSTRACT

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Lysozyme is an antimicrobial enzyme that could be applied to counteract those bacterial species which, due to their own metabolic activity, possess notable beer spoilage ability and lead to loss of beer quality. Experiments were carried out to assess lysozyme potential to prevent the growth of beer spoilage bacteria, and to verify the effect of lysozyme on the microbiological stability and sensory characteristics of unpasteurized beer. Eight replicates, all from the same lot of Italian beer, were treated with 0 and 100 ppm lysozyme. Microbiological analyses were conducted bimonthly to investigate the presence of spoilage bacteria. Sensory analyses were performed to determine whether there were any significant differences in sensory impressions between beers produced with and without lysozyme. Lysozyme exerted a strong inhibitory action on the lactic acid bacteria (LAB) present in the beer and was very stable throughout the shelf life. Sensory tests revealed no unfavourable influence on beer flavour when using lysozyme. Indeed, the shelf life of beer with added lysozyme proved to be extended. Even as late as 1 month after the expiry date it still met with the panellists' approval. Lysozyme may be regarded as an effective agent for preventing microbiological contamination and prolonging the stability of unpasteurized beer.

Key words: lactic acid bacteria (LAB), lysozyme, shelf life, unpasteurized beer.

INTRODUCTION

Although beer shows notable microbiological stability due to its physiochemical properties, certain bacterial species have been recognized as possessing potential beer spoilage ability. These contaminants can emerge during the malting and brewing processes, and consequently spoil the beer by their own metabolic activity, which leads to turbidity, acidity and undesirable off-flavours such as diacetyl or hydrogen sulphide. These changes have a negative impact on beer quality, with detrimental financial consequences for the brewing industry.

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The so-called beer spoilage microorganisms belong to both Gram-positive and Gram-negative bacteria, as well as to wild yeasts. Gram positive beer spoilage bacteria include several species of lactic acid bacteria (LAB), which are reported as the most hazardous bacteria for breweries as they are responsible for approximately 70% of all microbial spoilage incidents[3,31]. Indeed, LAB can be found in brewing materials at almost every stage of the malting and brewing process, from the standing barley crop to the finished beverage[13,17,28,33]. These bacteria occur as part of the natural barley micro-biota, and persist during malting and mashing[29,35] due to their aero tolerant nature and tolerance of low pH and high ethanol levels.

The genera *Lactobacillus* and *Pediococcus* are the predominant beer spoilage LAB species[20]. Among them the most important spoilage organisms are, according to brewing literature, *L. brevis, L. lindneri, L. paracollinoides and P. damnosus*[2,30,32,34]. In addition, *L. backi* and *P. inopinatus* have been reported as potential beer spoilers[7,22,33]. A recently characterised strain, *P. clausseni,* has been shown to spoil beer[12]. The strictly anaerobic *Pectinatus* and *Megasphaera,* showing characteristics of both Gram-negative and Gram-positive organisms, are commonly isolated from unpasteurized, packaged beer and brewery environments[15,16,25,27]. Important Gram-negative contaminants in the context of beer brewing belong to various *Enterobacteriaceae,* acetic acid bacteria, *Selenomonas,* and the strictly anaerobic bacteria *Zymomonas,* and *Zymophilus*[23,31].

Lysozyme (muramidase, EC 3.2.1.17) is an antimicrobial enzyme that can be applied against microbial growth in food and beverages[19]. It occurs in several mammalian secretions (milk, saliva, tears) and also in hen egg white, which represents the raw material of choice for the production of lysozyme on an industrial scale. Lysozyme is a fully natural product and no solvents are used to extract the protein from the albumen. This low molecular weight enzyme (14,400 Dalton), which consists of 129 amino acids cross-linked by four disulphide bridges, shows hydrolytic activity against the β(1→4) glycosidic bond between *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid in the cell wall of bacterial species, particularly Gram-positive organisms such as LAB[18]. In the cheese industry, its use is authorised as a bio-protectant to prevent butyric spoilage, which causes the late blowing of semi-hard cheeses by *Clostridium tyrobutiricum*[8,9]; in oenology it represents an alternative way to limit the proliferation of LAB spoilage bacteria during winemaking[5,14]. Its antibac-

terial spectrum is similar to what is described for nisin and hop acids, and its price is moderate.

Nisin is the best-known and most studied bacteriocin. It is produced by strains of *Lactococcus lactis* subsp. *lactis,* and it is the only one approved for food applications. Its numerous applications, as a natural food preservative, include dairy products, canned foods and processed cheese. Nisin is an effective bactericidal agent against Gram-positive bacteria and spores since it interferes with the cytoplasmic membrane of susceptible species, leading to pore formation, and dissipates the proton motive force[24]. However many Gram-positive bacteria have been shown to be resistant to it, due their ability to synthesize an enzyme, nisinase, which can inactivate the bacteriocin; moreover the cost of nisin addition to all fermentations would be prohibitively expensive[1,35].

Hop acids, mainly iso-α-acids such as iso-humulones, derive from the flowers of the hop plant. They exert bacteriostatic effects on most Gram-positive bacteria as they act as proton ionophores and dissipate the transmembrane pH gradient. Several LAB species, such as *Lactobacillus* spp., have been found to have acquired beer-spoilage ability due to hop resistance genes (*horA* and *horC*), and consideration must be given to the fact that altering the hop composition of beer can have a profound impact on the organoleptic and physico-chemical properties of the final product[6,21,22,33,35].

The aim of this study was to assess lysozyme potential to inhibit, or delay, the growth of spoilage LAB in naturally contaminated beer and, consequently, to test the effect of lysozyme on the microbiological stability and sensory attributes of unpasteurized beer. Of additional interest was the detection, isolation and identification of spoilage microorganisms commonly present in beer.

MATERIALS AND METHODS

Sampling

Samples of unpasteurized beer were collected from four European breweries: two in the Netherlands, one in Belgium and one in Italy, and 37 beer samples were investigated.

All the samples were transported to the laboratory and stored at room temperature, until microbiological and sensorial analysis.

Lysozyme effectiveness to prevent or delay growth of beer spoilage microorganisms

To assess lysozyme effectiveness against spoilage bacteria in unpasteurized beers of different origin, obtained through different production processes, six brewers were requested to set up the experimental tests. The samples were taken from the same production lot and were with or without lysozyme.

The lysozyme was added during the brewery bottling, and the samples were kept under normal storage conditions (i.e., those applied by the individual producers) and transferred to the laboratory after different periods of storage (Table I). Once transferred to the laboratory, the samples were subjected to analysis within 24 h.

The six brands of unpasteurized beers, each one including one sample with lysozyme (100–300 ppm lysozyme) and one or two samples without lysozyme, were analysed.

Two samples of yeast were also received in duplicate, with and without lysozyme (220 ppm lysozyme and 37.5 ppm nisin for the first one, 300 ppm lysozyme for the second).

A pH-meter (HI8418, Hanna Instruments, Woonsocket, Rl, USA) was used to measure the beer pH immediately before microbiological analysis. The pH electrode (FC200B, Hanna Instruments) was standardised using two buffers (pH 4.0 and pH 7.0). The pH was estimated as the mean value of two replicates for each sample.

For the microbiological control, conventional methods of incubation on culture media were applied.

Decimal dilutions of each previously homogenized sample were prepared in sterile Ringer's solution (Scharlau Microbiology, Barcelona, Spain) and plated in duplicate onto specific media for viable counts. For each of the

Table I. Lysozyme content and pH value of samples used in the study of lysozyme effectiveness on brewing spoilage microorganisms (L = lysozyme added).

Sample	Geographic origin	Source	Storage period (days)	Added lysozyme (ppm)	Determined lysozyme content (ppm)	pH
A	The Netherlands	triple fermented ale	206	-	<0.25	4.3
AL	The Netherlands	triple fermented ale	206	100	100	4.4
B	The Netherlands	triple fermented ale	206	-	<0.25	4.5
BL	The Netherlands	triple fermented ale	206	100	100	4.3
c_1	The Netherlands	top-fermented amber beer	10	-	< 0.25	4.2
c_2	The Netherlands	top-fermented amber beer	10	-	< 0.25	3.8
CL	The Netherlands	top-fermented amber beer	10	300	47	4.2
D	The Netherlands	bottom fermented ruby-red double malted beer	195	-	<0.25	3.6
DL	The Netherlands	bottom fermented ruby-red double malted beer	195	300	198	4.4
E	The Netherlands	bottom fermented ruby-red double malted beer	244	-	<0.25	4.3
EL	The Netherlands	bottom fermented ruby-red double malted beer	244	300	185	4.4
F	Italy	bottom fermented lager beer	30	-	<0.25	4.6
FL	Italy	bottom fermented lager beer	30	100	111	4.6
G	Belgium	brewing yeasts	9	-	<0.25	4.2
GL	Belgium	brewing yeasts	9	300	149	4.8

collected samples the following analyses were performed: LAB on De Man – Rogosa – Sharpe MRS medium[11] (Scharlau Microbiology) supplemented with Tomato Juice broth (10 g L^{-1}, Difco Laboratories, Detroit, MI, USA), natamycin (50 mg L^{-1}, DSM, Heerlen, The Netherlands) and agar (15 g L^{-1}, Scharlau Microbiology) under anaerobic conditions (Anaerocult A Merck, Darmstadt, Germany) at 30°C for seven days, yeasts on Rose Bengale agar (Scharlau Microbiology) at 25°C for five days, non LAB contaminating microorganisms on carbohydrate-free medium (ISO 13559:2002) at 30°C for 3 days. To prevent growth of yeasts, the medium was supplemented with natamycin at a concentration of 50 mg L^{-1} just before pouring the plates.

Study of beer spoilage microflora in samples of unpasteurized beer, brewing yeast and wort

After microbiological analysis, beer and brewing yeast samples previously examined and proven to be contaminated by LAB or non LAB spoilers were evaluated to determine the microflora present (Table II). Since the ingredients are considered serious sources for potential product spoiling microorganisms, four samples of wort were analysed. Wort samples comprised two saturated and two unsaturated worts. After counting, LAB and non LAB contaminant colonies were picked randomly from MRS agar and carbohydrate-free medium plates respectively. All the strains were subjected to purification on HHD agar (Biolife, Milano, Italy), a Gram stain and a catalase test. Cell morphology was examined by microscope. Working cultures were kept in MRS broth at 4°C before being submitted for phenotypic analysis. Presumptive identification of strains was carried out using the Biolog Microplate system (Biolog, Hayward, CA, USA) according to the manufacturer's instructions. AN (Anaerobic) MicroPlates were used for LAB, while GP (Gram Positive) Microplates were adopted for the non LAB contaminating microorganisms.

Effects of lysozyme on the microbiological stability and sensory attributes of beer

The samples of unpasteurized lager beer received from an Italian brewery, which presented natural contamination by *Lactobacillus brevis* and *L. malefermentans,* were used to study the effects of lysozyme on the microbiological stability and organoleptic attributes of beer. Eight replicates of the same lot of beer were treated with 0 and 100 ppm lysozyme. The microbiological profile of the two groups was compared. Microbiological and sensory analyses were conducted bimonthly, from the beginning of the storage period till 12 months after production. The beer used in this experiment had a 3-month shelf-life.

Lysozyme dosage

The lysozyme content in samples of beer and culture yeast was quantified by microbiological assay. The agar-plate method described by Lodi et al.[26] was adopted. Briefly, a medium composed of 1% agar dissolved in 0.1 M citrate buffer (pH = 6.2) was prepared; 15 mL of the medium was poured out in Petri dishes (9 cm diameter). After solidifying at room temperature, a second 5 mL agar layer containing 2 mg *Micrococcus lisodeikticus ATCC No. 4698* (Sigma, St. Louis, MO, USA) mL^{-1} was poured in. In each Petri dish 6 concentric, equidistant wells were made (9 mm diameter) and alternately filled with 120 µL of beer and lysozyme-added beer. For the lysozyme-added samples, 120 µL of a 0.1% solution of beer in 0.1 M citrate buffer (pH 6.2) was used. The same quantity of three solutions of standard lysozyme in 0.1 M citrate buffer (pH 6.2) containing 2, 4 and 8 µg mL^{-1} was employed to produce a calibration curve. These final solutions were obtained from a water solution containing 800 µg lysozyme hydrochloride mL^{-1} (Fordras S.A., Lugano, Switzerland).

Table II. Isolation and presumptive identification of brewing spoilage microorganisms from beer, brewing yeast and wort samples.

Origin	Source	Identification	Number of strains
NL[a]	triple top-fermented ale	*Micrococcus* spp.	1
NL	triple top-fermented ale	*Micrococcus* spp.	1
NL	bottom-fermented ruby-red double malted beer	*Staphylococcus* spp.	2
		Kocuria kristinae	1
NL	top-fermented amber beer	*Lactobacillus kefiri*	2
		Arcanobacterium pyogenes	1
		Staphylococcus epidermidis	1
		Staphylococcus warneri	1
		Micrococcus lylae	1
I[b]	bottom-fermented lager beer	*Lactobacillus brevis*	10
		Lactobacillus malefermentans	5
		Stenotrophomonas maltophilia	1
		Staphylococcus spp.	2
		Staphylococcus epidermidis	2
		Staphylococcus haemolyticus	1
		Staphylococcus hominis subsp. *novobiosepticus*	1
B[c]	brewing yeast	*Micrococcus* spp.	3
NL	saturated wort	*Leuconostoc mesenteroides subsp. mesenteroides*	1
		Wild yeast	2
NL	unsaturated wort	*Pseudomonas fluorescens*	2

[a] The Netherlands.
[b] Italy.
[c] Belgium.

All solutions were freshly prepared. The inoculated plates were incubated for 24 h at 35°C and the lytic diameters were measured. The lysozyme concentration in the beer samples was determined on the resulting calibration curve. This method was found to be suitable for measuring low levels of lysozyme with a quantification limit of 0.25 μg mL^{-1} beer.

Sensory analysis

To evaluate the appearance, taste and aroma of the Italian beer, a sensory analysis was performed.

Triangle tests (ISO 4120:2004) were carried out in order to determine any slight, but significant differences in sensory impressions between the beers produced with or without lysozyme. Thus, samples of beer were arranged in the shape of a triangle; they were prepared and identified by a three-figure number code and reported in a schedule. The samples were then evaluated at room temperature by a sensory panel (n = 12); neutralizers in the form of water and salt-free cracker biscuits were used between each tasting.

In addition, a descriptive sensory analysis, using appearance and flavour attributes, was carried out.

The visual examination considered 2 characteristics: froth (quantity and persistence) and appearance (qualitative evaluation); the assessment of olfactory intensity (quantity of odours) and olfactory fineness (quality of aroma) were made through smell. For gustatory, four characteristics were determined: fizziness (perception of carbon dioxide), body (sensation of structure), bitterness (intensity of bitter sensation) and retro-olfactory persistence (length of olfactory sensations in mouth). The panellists evaluated each feature as a score ranging from 1 to 5, the best being 5.

RESULTS

Lysozyme effectiveness to prevent or delay growth of beer spoilage microorganisms

All the beer and yeast samples investigated showed the presence of LAB, even if analysed at different months of their shelf life (Fig. 1), thus demonstrating the high frequency of LAB contamination in beer. The viable counts on MRS plates varied from 1.34 to 3.40 and from 0.50 to 2.49 $\log_{10}$ cfu mL^{-1} of beer for lysozyme non-treated and treated samples respectively. The LAB load reached the maximum detected value of 6.54 $\log_{10}$ cfu mL^{-1} in the sample of culture yeast without lysozyme. The control Italian beer, used to evaluate lysozyme effect on beer stability, also had 4.25 $\log_{10}$ cfu mL^{-1} viable cells at the beginning of the storage period (Table III).

There was a notable difference in LAB counts between the control and the lysozyme-added treatments. The LAB contamination level was lower in beers with added lysozyme. Lysozyme was thus demonstrated to be efficient in preventing LAB development.

Furthermore, the pH values stayed in the range of between 3.60 and 4.80 (Table I). Except for the triple fer-

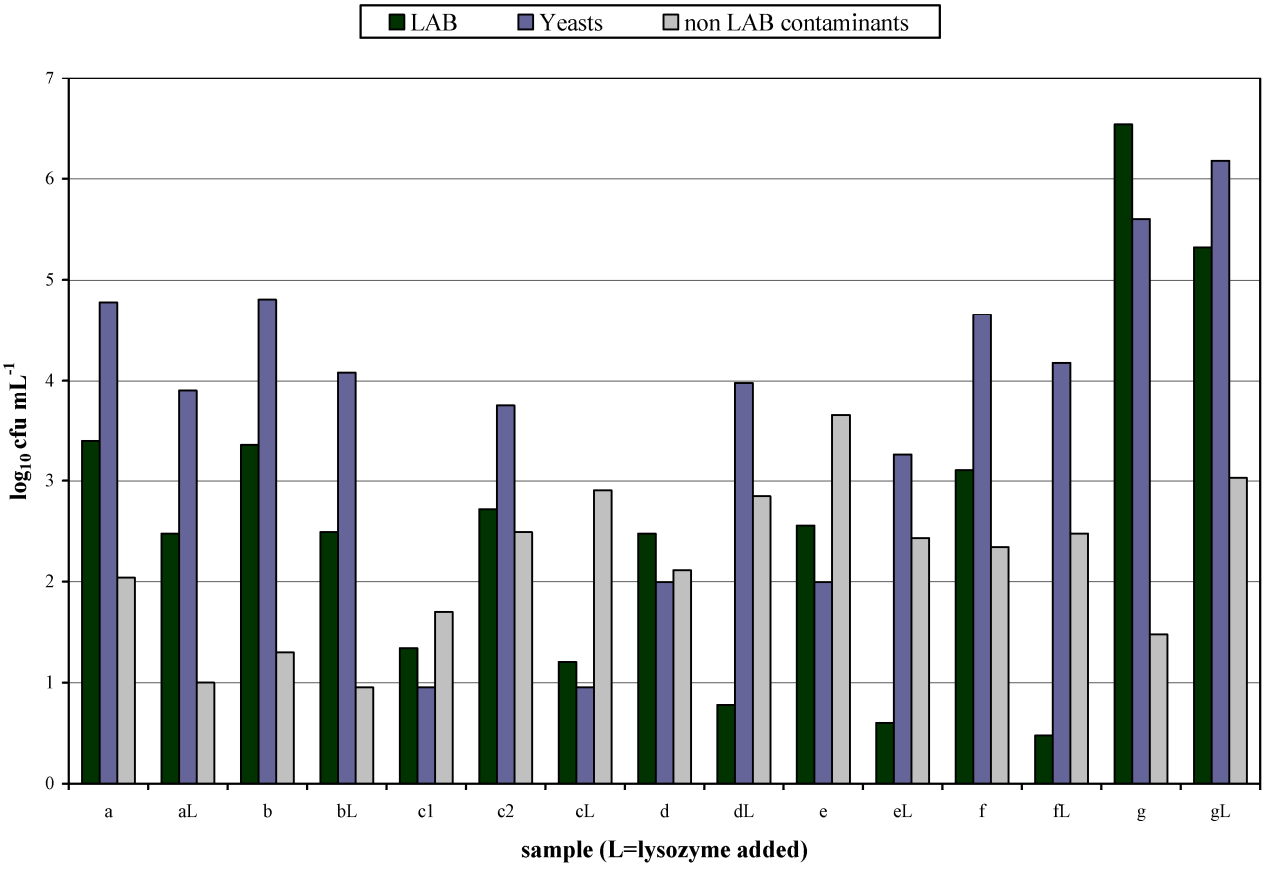

Fig. 1. Lactic acid bacteria (LAB), yeasts and non-LAB contaminating microorganisms found in the experimental samples.

mented beer (samples B and BL), higher acidity values were reached in the control samples. A higher LAB content corresponded with a decrease in pH, which can be attributed to the LAB acidifying capacity. With regard to non LAB contaminating microorganisms and yeasts, no correlation was observed between lysozyme presence or absence in beer and the bacterial population (Fig. 1).

Study of beer spoilage microflora in samples of unpasteurized beer, brewing yeast and wort

Altogether, 41 isolates were collected, 33 from beer samples and 8 from yeast and wort samples (Table II). Seventeen strains isolated from beer were Gram-positive, catalase-negative and belonged to the LAB. In particular, *L. brevis* (10 strains), *L. malefermentans* (5) and *L. kefiri* (2) were detected (Table II). Sixteen strains of non LAB contaminating bacteria were also isolated from beer. The most detected microorganisms were Gram-positive, catalase-positive bacteria belonging to *Staphylococccus* spp. (9 strains) and *Micrococcus* spp. (3). In particular, isolates included *Staphylococcus epidermidis* (2), *S. warneri* (1), *S. haemolyticus* (1), *S. hominis* subsp. *novobiosepticus* (1), and *Micrococcus lylae* (1). But *Kocuria kristinae* (1), *Arcanobacterium pyogenes* (1) and the Gram-negative bacterium *Stenotrophomonas maltophilia* (1) were also found.

The results showed that in the sample of pitched brewing yeast supplemented with 220 ppm lysozyme and 37.5 ppm nisin, no contamination occurred, while the sample of yeast without lysozyme had cocci belonging to *Micrococcus* spp. (3 strains).

Samples of unsaturated wort were infected with *Pseudomonas fluorescens* (2 strains).

Both samples of saturated wort were contaminated by wild yeasts (2 strains). The lactic acid bacterium *Leuconostoc mesenteroides* subsp. *mesenteroides* (1 strain) was detected in one sample of saturated wort.

Effects of lysozyme on the microbiological stability and sensory attributes of beer

In order to test the efficacy of lysozyme under real conditions, two series of naturally contaminated samples, with and without lysozyme addition, were examined throughout the storage period. Moreover in validation studies, for the detection and enumeration of LAB in beer, naturally contaminated samples are preferred to artificially contaminated ones (EN ISO 16140:2008).

Lysozyme stability in the beer was determined throughout the experiment; eight samples (BL) were subjected monthly to lysozyme detection by the agar plate method. Table III shows the small differences found among the replicates. Indeed, the amounts of lysozyme ranged from 81 to 100 ppm. Lysozyme dosage showed that the enzyme proved to be constant. A slight loss was found on analysing the beer after 8 months from production, when the amount was lower (81 ppm): however the recovery of lysozyme activity from beer was 93% on average. Lysozyme content was also evaluated in the corresponding, naturally contaminated, non-treated beers (B), thus confirming the absence of the enzyme in those samples.

Even though the yeasts showed a natural, slow decrease throughout the experiment, the lysozyme did not affect the yeast, as the yeast counts were often higher in treated samples.

Similarly, non LAB contaminating microorganisms did not prove to be sensitive to lysozyme. In the treatments containing 100 ppm lysozyme, the contaminant microflora suffered a reduction in the cell count within 2 months of storage. However, the same samples showed an increase in number of non LAB contaminants around the middle of shelf life.

There was however strong lysozyme inhibition activity on the LAB population. The LAB (*L. brevis and L. malefermentans*) naturally present in beer were demonstrated to be sensitive to lysozyme at a dosage of 100 ppm. In particular, *L. brevis* sensitivity, previously highlighted in beer[10] and wine[14], was confirmed. The LAB viable counts in the control beer varied from 3.88 to 5.83 $\log_{10}$ cfu mL^{-1}, while 1.30 $\log_{10}$ cfu mL^{-1} was the maximum load detected in the lysozyme-added samples. The results demonstrated that beer with lysozyme added was stable for the storage period (1 year).

Since natural lactic acid fermentation causes the acidification of beer, the pH values were generally found to be lower in the control samples than in the corresponding treated samples, ranging from 3.9 to 4.6 for the non-treated samples (B) and 4.3 to 4.6 for the lysozyme treated samples (BL).

As revealed by the triangle difference method of sensory analysis, treatment with lysozyme did not affect the

Table III. Lysozyme content, pH value and microbial counts of beer during shelf-life (L = lysozyme added).

Shelf-life (months)	Lysozyme content (ppm)		pH		LAB ($\log_{10}$ cfu mL^{-1})		Yeast ($\log_{10}$ cfu mL^{-1})		Non-LAB bacteria ($\log_{10}$ cfu mL^{-1})	
	B[a]	BL[b]	B	BL	B	BL	B	BL	B	BL
0	<0.25	108	4.60	4.60	4.25	0.48	4.83	4.26	1.48	1.00
1	<0.25	88	4.30	4.50	5.83	0.70	4.02	4.53	1.30	1.00
2	<0.25	98	4.30	4.50	5.55	0.00	3.95	3.48	1.00	1.00
4	<0.25	99	4.30	4.60	3.88	1.30	1.00	3.82	1.00	1.30
6	<0.25	93	4.20	4.40	5.13	0.30	<1	<1	<1	1.85
8	<0.25	81	4.10	4.40	5.40	1.28	<1	2.97	2.58	2.90
10	<0.25	100	3.90	4.30	5.73	0.00	2.15	1.60	2.20	2.08
12	<0.25	85	4.00	4.50	4.83	0.00	2.64	2.96	2.94	1.30

[a] Control beer.
[b] Lysozyme-treated beer.

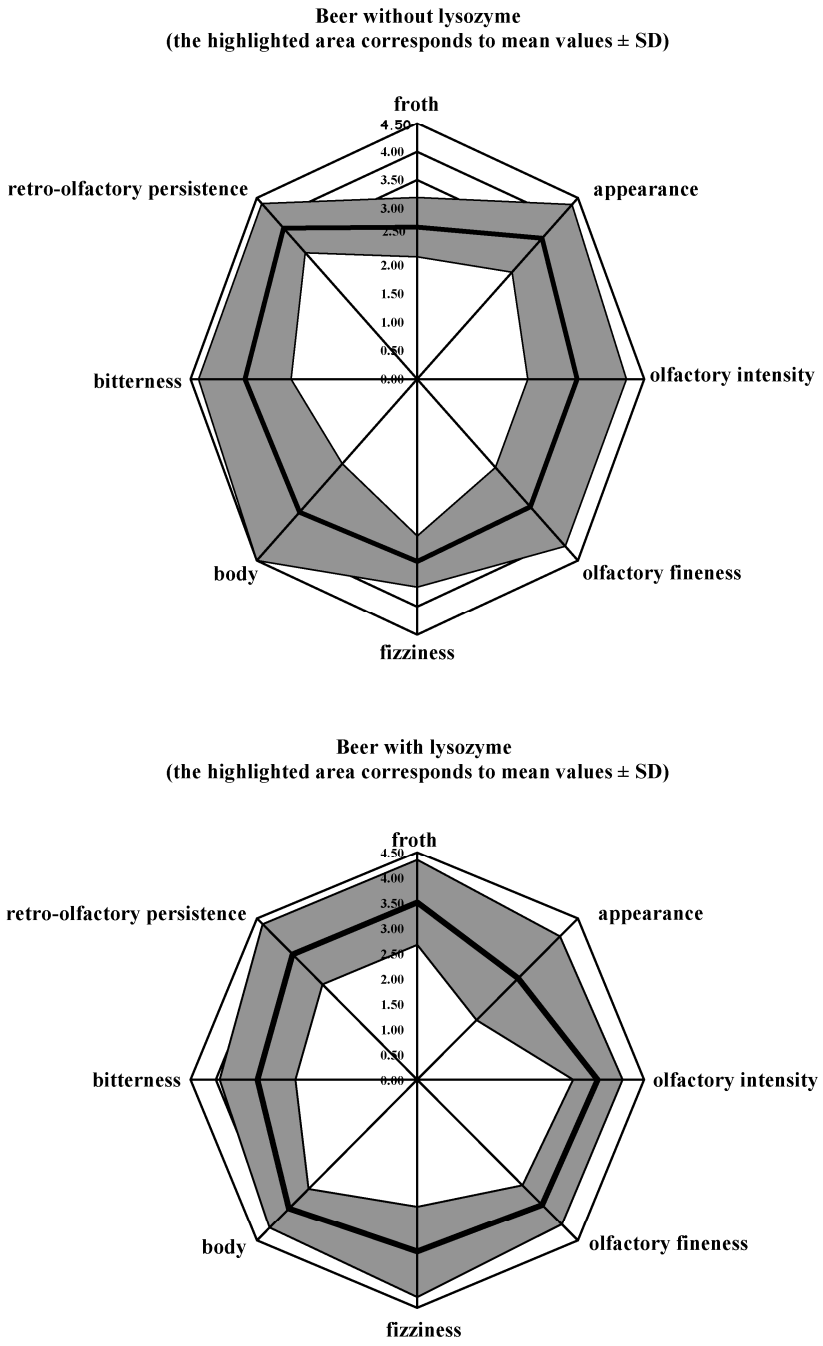

Fig. 2. Sensory profile of beer with and without the addition of lysozyme.

organoleptic attributes of the beers tested throughout the 12 month storage period. Sensory analysis (with 12 panellists) carried out 1 month from production, concluded that the two beers were no different at the 5% significance level. Conversely, as late as 1 month after the expiry date (4 months from production) the two beers were noted as different at the 5% significance level. Furthermore, the sensory panel noted a pleasing, mellower flavour in the beer with added lysozyme. The descriptive sensory analysis revealed a major persistence of the froth in the lysozyme-added beer. This beer was also rated as less bitter and with a perception of fizziness (Fig. 2). The beer without lysozyme, despite of the presence of beer spoilage LAB, did not present significant changes in turbidity, acidity or off-flavour as might have been expected[4].

DISCUSSION

The unpasteurized beers presented notable contamination by LAB, though there was no negative change in beer quality observed. Other bacteria implicated in beer spoilage such as *Pediococcus, Megasphaera* and *Pectinatus*[35] were never detected.

The results of the present research indicate that lysozyme can play a role in prolonging the stability of unpasteurized beer because of its strong inhibitory action on

LAB growth. Sensory tests proved that the use of lysozyme did not lead to any unfavourable influence on the flavour of the beer. Indeed, the shelf-life of the beer with added lysozyme proved to be extended to beyond the expiry date. The beer still met with the panellists' approval, and had, according to them, a more appreciated flavour. Previous studies have reported similar results. Experiments conducted at Oregon State University, to evaluate possible lysozyme effects on the physical and sensorial properties of beer, led to an assessment by Daeschel et al.[10] that to a maximum concentration of 200 ppm, lysozyme did not cause chill haze and did not affect foam stability. Furthermore, two sensorial studies performed by both a regular consumer panel and a brewing industry professional panel, also demonstrated that lysozyme had no impact on the flavour of beer. In fact, neither panel could detect any difference in lysozyme treated beers. Moreover, lysozyme added to beer showed strong stability throughout the experiment[10].

In conclusion, our study confirms a new application of lysozyme for prolonging the shelf-life of unpasteurized beer. Further investigations will be made into the relationship between the use of lysozyme and the improvement noted in beer flavour and texture. In addition, studies are still required to determine how lysozyme affects the growth of yeasts. It should be remembered that, at present, there is no data available on the possibility of LAB acquiring resistance to lysozyme.

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