International Food Research Journal 19(2): 783-786 (2012)

Short Communication
The effect of antimicrobials and bacteriocins on beer spoilage microorganisms

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Abstract: This study investigated the effect of lysozyme, nisin, sakacin and hope extract on beer spoilage microorganisms and determined the minimum inhibitory concentrations (MIC) for them. The results indicated no efficacy of any agent against yeasts and *Acetobacter aceti* and only nisin and lysozyme presented action against the Lactic Acid Bacteria (LAB) evaluated. The highest MICs were determined to *Lactobacillus brevis*, which required 3 mg L^{-1} and 100 $mg.L^{-1}$ of nisin and lysozyme, respectively, to its inhibition. The evaluation of *L. brevis* exposure to lysozyme during 10 days indicated that the microbial reduction had continued. The results of this research showed that lysozyme and nisin could be used to control some LAB contamination in beer, but need to be associated to other method to guarantee the stability of beer.

Keywords: Beer, dynamic high pressure, lactic acid bacteria, antimicrobials

Introduction

Pilsen is the most consumed type of beer in the Brazilian market. It is a clear, light yellow beer with alcoholic content of about 4.5ºGL, and with good foam retention. The main beer contaminants genera are lactic acid bacteria (*Lactobacillus* e *Pediococcus*) and acetic (*Acetobacter* e *Acetomonas*), *Pectinatus, Zymomonas, Megasphaera*, *Micrococcus* and yeasts (Vaughan *et al.,* 2005). Among the contaminants, *L. brevis* is distinguished by producing off-flavors and turbidity in beer (Priest and Campbell, 1999; Asche, 2000).

The beer is industrially packaged in metal cans or glass bottles and pasteurized to ensure the microbiological stability (Zuffall and Wackerbauer, 2000). This pasteurization, however, promotes undesirable changes in beer, as the denaturation of proteins with consequent increase of tannin-proteins complexes (Stewart, 2006), the increase of the red colour as a consequence of products of Maillard reaction (Castellari *et al.*, 2000), the formation of undesirable odours of paper and cardboard due to oxidation (Furucho *et al.*, 1999) and the increased bitterness by the α-acids isomerisation (Wackerbauer and Zufall, 1998).

Thus, the breweries have been searching for new methods of microbiological stabilization to maintain the beverage sensory quality. Among these new methods, the application of bacteriocins and antimicrobials was successfully tested either as inhibitor or to delay growth of some beer contaminants (Chihib *et al.*, 1999; Silvetti *et al.*, 2010), with shelf life increases of up to a month (Silvetti *et al.*, 2010). Considering these previous data, the objective of this research was to determine the antimicrobial and bacteriocins effects on the main beer contaminants.

Materials and Methods

Bacteria and growth medium

Lactobacillus brevis (CCT 3745), *Lactobacillus delbrueckii* (ATCC 9649), and *Acetobacter aceti* (CCT 2565) were obtained as a donation from the Tropical Culture Collection / Tropical Research and Technology Foundation (Campinas, São Paulo, Brazil). *Listeria innocua* (LH 475) was obtained as a donation from Hygiene Laboratory of School of Food Engineering of State University of Campinas (UNICAMP, Campinas, São Paulo, Brazil). *Schizosaccharomyces ludwigii* and *Saccharomyces diastaticus*, were obtained from a particular culture collection of a Brazilian brewery (Ambev, Jacareí - São Paulo, Brazil). A *Lactobacillus sakei* 2a recognized as sakacin producer was obtained from Laboratory of Food Microbiology of School of Pharmaceutical Science of University of São Paulo (USP, São Paulo, Brazil).

Lactic acid bacteria (LAB) were grown in Oxoid ® *Lactobacillus* media (MRS - De Man, Rogosa, Sharp - agar and broth). Acetic bacteria was grown in MRS and 2% ethanol and the yeasts were either grown in potato dextrose agar (Oxoid®) or malt extract broth enriched with 4% w/v of yeast extract (Oxoid®). *Listeria* grew in Oxoid® Tryptone Soy Broth (TSB) and agar (TSA) (Oxoid®). *A. aceti* was preserved in

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MRS added by 2% of ethanol and the other bacterial strains were preserved in Litmus milk media (Difco®). Yeasts were preserved in PDA covered with sterile mineral oil. Cultures of 24 h were used in the tests of microbial reductions.

Antimicrobials and nisin

Lysozyme and nisin were obtained from the commercial formula, respectively Novagard® and Nisaplin® from Danisco S.A.S. The hope extract was obtained from Rhodia, S.A.S.

The sakacin was produced by *L. sakei* 2a by fermentation. The media was prepared as described by Liserre *et al.* (2002), added by 0.5% glucose to minimize the pH reduction. After fermentation, the broth (extract) was micro filtered in 0.22 µm filter and had the pH adjusted to 6.0 using sodium hydroxide 0.1M – to prevent microbial inhibition. The bacteriocin production was determined by the agar diffusion assay, as described by Rogers and Montville (1991). Test was performed in triplicate.

Determination of minimum inhibitory concentration (MIC)

The MIC determination was performed by method described by Tribst *et al*. (2008). It were tested concentrations of nisin ranging from 0.5 to 200 mg. L^{-1}, of lysozyme from 0.5 to 1000 mg. L^{-1}, of hops extract between 10 and 1000 mg. L^{-1} and of sakacin from 1 to 50% of the extract obtained by the *L. sakei* 2a fermentation. Tests were conducted in triplicate.

Determination of L. brevis *growth in beer added by lysozyme*

Pasteurized Lager beer (pH 4.5, 4.7°GL, and Brix 2.5) was obtained from a Brazilian brewery. In order to inactivate natural biota, the beer sterilized at 121ºC/15 min. Then, the beer was added with lysozyme at concentration of 50 $mg.L^{-1}$ and after intentionally inoculated with 1% (v/v) of a *L. brevis* suspension (10^8 $CFU.mL^{-1}$). Microbial counts were performed in MRS agar in the first's hours of inoculation and during 10 days. Similar test was performed using phosphate buffer (pH 6,0). Tests were performed in triplicate.

Results and Discussion

The results showed that the yeasts were not inhibited under the tested conditions, indicating that these substances are not effective in controlling these microorganisms in beers. Such observation was expected, since none of the tested compounds had been previously reported to be effective against yeasts and molds (Bhattacharya *et al.*, 2003; Carvalho *et al.*, 2010). This indicates that the use of antimicrobials and bacteriocins to preserve beer must to be associated with other preservation methodology, able to reduce the yeasts on beer.

Also, no reduction was observed to *A. aceti*. Again, it was expected, since other authors previously reported the low efficacy of these antimicrobials and bacteriocins against Gram negative bacteria (Bhattacharya *et al*., 2003; Carvalho *et al.*, 2010). Considering a beer preserved with antimicrobials and bacteriocins, no effect will be observed in *A. aceti* population, however, taking into account that the *A. aceti* only grow and produce acetic acid in strictly aerobic conditions (Ory *et al*., 1998), it was possible to control this microorganism with an effective anaerobiosis, which is required in beer to prevent the oxidation of its constituents and formation of undesired flavors (Furucho *et al*., 1999).

The evaluation of LAB results indicates that they are susceptible to nisin and lysozyme and that *L. brevis* was the microorganism higher resistant. Also, it was observed that nisin was more effective, requiring low concentration (3 $mg.L^{-1}$) for LAB inhibition. Previous results have demonstrated the nisin effectiveness in promoting lag phase increase and growth rate reduction (Chihib *et al*., 1999) as well to inactivate contaminants in beer (Galvagno *et al.*, 2007), corroborating the potential use of this bacteriocin. High concentration of lysozyme was need to inhibit the growth of the tested microorganisms and, to *L. brevis*, the inhibition was transitory at 50 $mg.L^{-1}$, being need the use 100 $mg.L^{-1}$ to obtained a condition that permanent inhibited the LAB growth. Prior results demonstrated that lysozynme is not only able of slowing (Makki and Durance, 1996) or inhibiting (Silvetti *et al.*, 2010) the growth of beer contaminants, but also to promote the inactivation of existing microorganisms (Silvetti *et al*., 2010), increasing the shelf life of not pasteurized beer in a month. The MIC of antimicrobials and bacteriocins are shown in Table 1.

Table 1. MIC of antimicrobials and bacteriocins on beer spoilage microorganisms

Microorganism	Nisin ($mg.L^{-1}$)	Lysozyme ($mg.L^{-1}$)	Hope Extract ($mg.L^{-1}$)	Sakacin (% of extract - v/v)
L. brevis	3.0	50.0*	>1000.0	>50%
A. aceti	>100.0	>1000.0	>1000.0	>50%
L. delbrueckii	0.8	1.0	>1000.0	>50%
S. ludwiggi	>200.0	>200.0	>1000.0	>50%
S. diastaticus	>200.0	>200.0	>1000.0	>50%

* Transitory inhibition reversed after 48 h of incubation, permanent inhibition was observed at lysozyme concentration of $100mg.L^{-1}$

Considering the antimicrobials and bacteriocins studied, only the nisin and lysozyme were effective against some of the microorganisms evaluated. The

hops extract, although previously reported as being effective in inhibiting the *Lactobacillus* (Hammond *et al*., 1999) and other possible beer contaminants such as Pediococcus (Galvagno *et al.,* 2007), was unable to control the growth of any of the evaluated species at concentrations up to 1 gL^{-1}, demonstrating inadequacy in controlling beer contaminations, since higher concentrations would leave an undesirable flavor in the beer. Previous results had shown that *L. brevis* can be extremely resistant to hops extract (Sakamoto and Konings, 2003), through of protective mechanisms that prevents the influx of hops active compounds into the cells. Sakacin production was detected through the inhibition of *Listeria innocua* growth (data not shown), however, this bacteriocin did not affected the beer microbial contaminants. Results obtained by Vaughan *et al.* (2001), reported that sakacin effectiveness is a function of different species of *L. sakei* used for the bacteriocin production, thus, better results might be obtained with other subspecies of *L. sakei*.

The effect of lysozyme addition in beer to inhibit *L. brevis* was evaluated. The results indicated a low reduction in the initial load after 2 h of contact (0.5 log reduction) but around 4.5 reductions after 10 days of beer storage at room temperature (25°C). The evaluation of the same test made in phosphate buffer (pH 6,0) showed that lysozyme was active just in the two first hour of contact, with around 1 decimal reduction of *L. brevis*. These results indicated that the *L. brevis* inhibition by lysozyme was improved in beer, probably due to beer characteristics as low pH and alcohol concentration.

Conclusion

From the obtained results it was concluded that only the nisin and lysozyme were active on Gram positive beer contaminants at concentration of 3 and 50 mg L^{-1}, respectively. However, it is important to observe that no preservative evaluated were able to inhibit all beer contaminants. It highlight the need of association of other preservative method to nisin and lysozyme to guarantee the beer stability during its storage.

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