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Exploring the use of natural antimicrobial agents and pulsed electric fields to control spoilage bacteria during a beer production process

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ABSTRACT

Different natural antimicrobials affected viability of bacterial contaminants isolated at critical steps during a beer production process. In the presence of 1 mg/ml chitosan and 0.3 mg/ml hops, the viability of *Escherichia coli* in an all malt barley extract wort could be reduced to 0.7 and 0.1% respectively after 2 hour- incubation at 4 °C. The addition of 0.0002 mg/ml nisin, 0.1 mg/ml chitosan or 0.3 mg/ml hops, selectively inhibited growth of *Pediococcus* sp. in more than 10,000 times with respect to brewing yeast in a mixed culture. In the presence of 0.1mg ml chitosan in beer, no viable cells of the thermoresistant strain *Bacillus megaterium* were detected. Nisin, chitosan and hops increased microbiological stability during storage of a local commercial beer inoculated with *Lactobacillus plantarum* or *Pediococcus* sp. isolated from wort. Pulsed Electric Field (PEF) (8 kV/cm, 3 pulses) application enhanced antibacterial activity of nisin and hops but not that of chitosan. The results herein obtained suggest that the use of these antimicrobial compounds in isolation or in combination with PEF would be effective to control bacterial contamination during beer production and storage.

Key words: beer spoilage bacteria, brewing yeast, natural antimicrobials, pulsed electric fields

RESUMEN

Exploración del uso de agentes antimicrobianos naturales y de campos eléctricos pulsantes para el control de bacterias contaminantes durante el proceso de elaboración de cerveza. Diferentes antimicrobianos naturales disminuyeron la viabilidad de bacterias contaminantes aisladas en etapas críticas del proceso de producción de cerveza. En un extracto de malta, el agregado de 1 mg/ml de quitosano y de 0,3 mg ml de lúpulo permitió reducir la viabilidad de *Escherichia coli* a 0,7 y 0,1%, respectivamente, al cabo de 2 horas de incubación a 4 °C. El agregado de 0,0002 mg/ml de nisina, 0,1 mg/ml de quitosano o de 0,3 mg/ml de lúpulo inhibió selectivamente (10.000 veces más) el crecimiento de *Pediococcus* sp. respecto de la levadura de cerveza en un cultivo mixto. El agregado de 0,1 mg/ml de quitosano permitió disminuir la viabilidad de una cepa bacteriana termorresistente, *Bacillus megaterium*, hasta niveles no detectables. Por otra parte, el agregado de nisina, quitosano y lúpulo aumentó la estabilidad microbiológica durante el almacenamiento de cervezas inoculadas con *Lactobacillus plantarum* y *Pediococcus* sp. aislados de mosto de cerveza. La aplicación de campos eléctricos pulsantes (CEP) (3 pulsos de 8kV/cm) aumentó el efecto antimicrobiano de la nisina y del lúpulo, pero no el del quitosano. Los resultados obtenidos indicarían que el uso de antimicrobianos naturales en forma individual o en combinación con CEP puede constituir un procedimiento efectivo para el control de la contaminación bacteriana durante el proceso de elaboración y almacenamiento de la cerveza.

Palabras clave: bacterias contaminantes de cerveza, levadura de cerveza, antimicrobianos naturales, campos eléctricos pulsantes

INTRODUCTION

Beer is not a good environment for microbial growth because ethanol and hop compounds levels can be high enough to make beer bacteriostatic or bactericidal (26). Furthermore, the low pH and lack of fermentable sugar and oxygen inhibit growth of pathogenic and most non-pathogenic bacteria. Nevertheless, bacterial contaminants can be traced along the whole brewing process and the so-called beer spoilage microorganisms can cause an increase in turbidity and unpleasant organoleptic changes in beer through the elaboration and storage process (10).

Bacteria are commonly considered a minor problem in the wort to be fermented provided that it is promptly pitched. However, it has been established that coliforms are able to continue their growth after yeast has begun to multiply and the presence of their metabolic products in beer can adversely modify its taste and aroma (19, 26).

Another source of contamination usually comes from pitching yeast. Gram (-) genera and Lactic Acid Bacteria (LAB) are frequently detected and they can be more than 1% of the cell number of yeast inoculum (20). Brewing conditions naturally select for LAB and their growth causes haze, acidity and unpleasant flavor changes in beer (32). LAB are the major potential spoilage microorganisms in beer at the fermentation stage. Their growth leads to a beer with too much developed acid and off- flavors due to diacetyl production (9). Finally, can or glass bottle filler systems can be another possible contribution to beer contamination. At the brewery, fillers can be reservoirs of sporulated thermotolerant bacteria such as *Bacillus* spp. Conventional time-temperature combinations applied during pasteurization are not effective in eliminating these heat resistant microorganisms without affecting the organoleptic properties of beer or bottle integrity.

Natural antimicrobials have been shown to provide an efficient way of reducing or eliminating bacterial contamination of beers (6, 22, 26). Among them, the heat-stable peptide nisin has been used as an effective antimicrobial agent against beer-spoilage LAB (21, 32). Like most bacteriocins, it has a limited activity spectrum, being normally active against gram (+) but not against gram (-) bacteria, yeasts or molds (2, 8, 14). Nisin has no effect on intact yeast due to specific cell wall proteins that prevent the bacteriocin access into the cytoplasmatic membrane (3).

Hops is one of the main beer components. Particularly, the a-acids (humulones) and their isomerization products (isohumulones) confer flavor, bitterness, foam stability and antimicrobial activity to the finished beer. Studies on the antiseptic properties of hopped wort and hop boiling products showed that these compounds are specifically toxic for gram (+) bacteria (4, 28). It has been reported that beer-spoiling LAB can possess a plasmid-encoded hop resistance mechanism, HorA, which mediates an ATP-dependent efflux pump of hops that would be a prerequisite for bacterial growth in beer (27).

Chitosan is a biopolymer derived from chitin deacetylation obtained mainly from crustaceous shells. The antimicrobial activity of chitosan and its derivatives is exerted against different groups of bacteria and fungi (11, 17, 18, 29). Several mechanisms for the antimicrobial action of chitosan have been reported (25, 29, 31). Among them, it has been proposed that the positively charged amines of this glucosamine polymer, could interact with the negatively charged residues of macromolecules (mainly proteins) on the cell surface of bacteria and fungi. Thus, a layer that prevents the uptake of different substrates of the medium, is formed (24). A selective antimicrobial activity against LAB isolated during beer production has been previously reported in our laboratory (6).

On the other hand, Pulsed Electric Fields (PEF) have the potential to replace or complement conventional thermal pasteurization methods by producing only a small increase in temperature and minimizing organoleptic changes in foods. In particular, the application of PEF for beer preservation could prevent excessive darkness and overcooked flavor. It has also been reported that PEF treatment in the presence of nisin or hops can increase the inactivation of *Lactobacillus plantarum* (34) and gram (-) bacteria (13, 23, 33), therefore expanding their spectrum of action. This could be explained by the proposed mechanisms of action of nisin, hops and PEF. For all of them, the primary site of action is the cytoplasmic membrane which alters its selective permeability and causes the cessation of critical biosynthetic processes (22, 32, 34).

The overall objective of this work was to compare the effectiveness of nisin, chitosan and hops, in isolation or in combination with PEF. These effects were tested against representative bacterial contaminants isolated at various stages during actual brewing processes.

MATERIALS AND METHODS

Microorganisms and media

Lactobacillus plantarum and *Pediococcus* sp. were isolated in a local microbrewery from an all malt-barley- extract wort [AME, (10 °B, pH= 5.8 ± 0.1)] commonly used for beer lager production. *L. plantarum* was grown in De Man, Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, France) and *Pediococcus* sp. in Briggs tomato juice broth.

E. coli was isolated from an unpitched brewing wort and grown in Luria-Bertani medium. *Bacillus megaterium* was isolated from beer fillers. Both species were provided by a local industrial brewery.

A commercial lager yeast strain of *Saccharomyces cerevisiae* (“Lager 2247 European”, Wyeast Laboratories, Inc., Mt Hood, OR 9704, USA) was provided by a local microbrewery. Yeast cells were grown in Sabouraud broth plus yeast extract (0.5% wt/vol). Media were solidified with 1.5% (wt/vol) agar when required.

L. plantarum, *E. coli*, *Pediococcus* sp. and *B. megaterium* were identified by morphological and biochemical tests; *L. plantarum* identity was confirmed by the *API* 50CHL System (bioMérieux, Marcy L'Étoile, France). Strains were maintained in vials in the corresponding growth medium with 30% (wt/vol) glycerol at –70 °C.

Commercial beer containing ca 16-18 ppm a-isoacids, was locally acquired.

Assay for antimicrobial activity

Microbial inocula were grown in the appropriate liquid media for 12 h at 28 °C (*Pediococcus* sp.) or at 37 °C (*E. coli*, *B. megaterium* and *L. plantarum)* and 18 h at 28 °C (yeast). Then, cells were collected by centrifugation at 5,600 x g for 15 min (bacteria) or 5 min (yeast), washed and suspended in the corresponding medium (AME or beer) containing the antimicrobial agent.

After the assay, cell viability was assessed by spotting 20 µl of an appropriate dilution in peptoned water (0.1% wt/vol) on Petri dishes containing the corresponding solid medium for each microorganism. Plates were incubated 24-48 h at 28 °C (*Pediococcus* sp.) or 37 °C (*L. plantarum*, *E. coli* and *B. megaterium*) and 48-72 h at 28 °C (yeast). The viability of mixed bacteria/yeast samples was determined on Briggs tomato juice agar with cycloheximide (50 mg/l) or in yeast extract glucose chloramphenicol agar (YGC) (Biokar Diagnostics, France) to select for bacteria and yeast respectively.

Results were expressed as Colony Forming Units per ml (CFU/ml).

Antimicrobial agents

Pure nisin (Aplin & Barrett Ltd, UK) was generously provided by AMG SRL, Argentina. A 0.25 mg/ml (10,000 UI/ml) stock solution of nisin in distilled water at pH 4.5 was prepared and maintained at –20 °C.

Chitosan (930 kDa) was a generous gift from Dr. F. Shahidi (Memorial University of Newfoundland, Canada). A stock solution of 1% (wt/vol) chitosan in 1% (vol/vol) acetic acid was freshly prepared before using.

An isomerized hop extract *Isohop* (alkaline aqueous solution of a-isoacids 30% wt/wt), was provided by Haas Hop Products INC (USA) and maintained at 4 °C. Aliquots of adequate volumes of the extract were added to wort, AME or beer, according to supplier's technical specifications to attain the desired concentration of hop extract.

Appropriate aliquots of the antimicrobials stock solutions were added aseptically to the assay media. For each tested microorganism, a control experiment was set up, an equal concentration of cells were suspended in the solvent stock solution but with no antimicrobial added and similarly incubated.

PEF were carried out using a Gene Pulser II system (Bio-Rad). Aliquots of 400 µl of the bacterial suspensions were withdrawn into 0.2-cm electrode gap electroporation cuvettes (Bio-Rad Laboratories, 2000 Alfred Novel Drive, Hercules, CA 94547) and 3 pulses of an electric field intensity of 8 kV/cm^{-1} (50 µF capacitance) were applied. PEF conditions were established according to previous results from our laboratory (data not shown) in order to obtain a sublethal effect on the microorganism studied. After pulsing, samples were immediately placed in ice water, and analyzed within 1 hour.

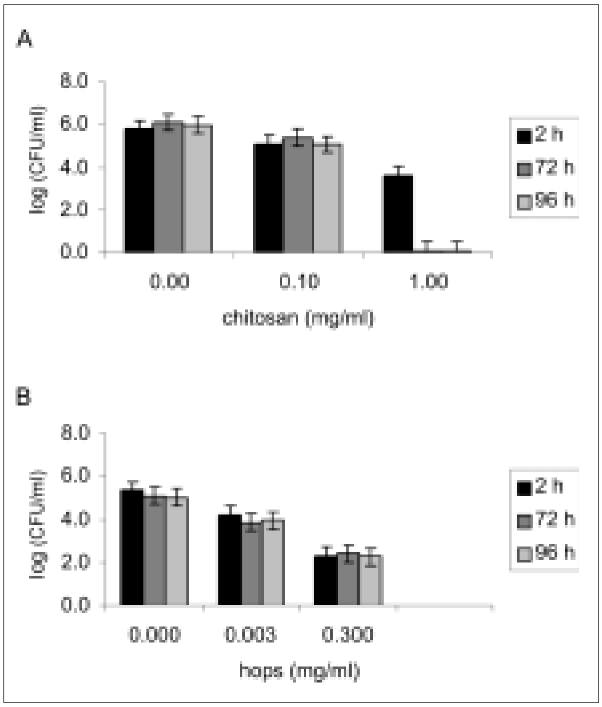

Figure 1. Inhibitory effect of (A) chitosan and (B) hops on *E. coli* viability during incubation in AME at 4 °C. Data are expressed as mean ± SD (*n* = 3).

RESULTS AND DISCUSSION

1. Effect of the antimicrobials on a bacterial contaminant isolated from unpitched wort

The inhibitory effect of chitosan and hops in isolation or in combination with PEF on a *E. coli* strain was studied. The antimicrobial effect of the compounds tested was determined after different contact times with bacterial cells. Figure 1A shows that the highest concentration of chitosan assayed (1 mg/ml), caused a 2 logarithmic cycle reduction (0.7% survival) after two hours incubation in AME at 4 °C, while no viable cells were detected after 72 and 96 h. These results indicate that a significant antibacterial effect of chitosan on *E. coli* at concentrations as high as 10^6 CFU/ml, could be achieved after a short contact time. Simultaneous PEF application did not improve the inhibitory effect of chitosan (data not shown).

Figure 1B shows the antimicrobial effect of hops up to 0.3 mg/ml on *E. coli* cells after different periods of incubation. As can be seen, independently of contact times, viability decreased by 1 logarithmic cycle in the presence of 0.003 mg/ml hops, while a 3 logarithmic cycle reduction (*ca*. 0.1-0.2% survival) was observed in media containing 0.3 mg/ml hops. PEF treatment in the presence of 0.3 mg/ml hops, increased viability reduction by up to 4 logarithmic cycles as it is shown in Table 1.

Ulmer *et al.* (34) informed that PEF application increased the inhibitory action of hops on a gram (+) beer spoiling strain of *L. plantarum*. In our experiments, we found a similar result when studying a gram (-) bacteria, reported as more resistant to antimicrobial treatments (28).

Table 1. Effect of PEF on antimicrobial activity of hops on *E. coli* after 2 h incubation in AME at 4 °C.

Hops (mg/ml)	log (CFU/ml)[1]	
	-PEF	+PEF
0.0000	5.4 ± 0.4 (0.0)[2]	2.4 ± 0.3 (3.0)
0.0003	5.3 ± 0.5 (0.1)	2.3 ± 0.4 (3.1)
0.0030	4.2 ± 0.6 (1.2)	2.2 ± 0.6 (3.2)
0.0300	4.2 ± 0.4 (1.2)	1.5 ± 0.2 (3.9)
0.3000	2.3 ± 0.3 (3.1)	1.0 ± 0.2 (4.4)

[1]Data are the average of at least three separate experiments ± standard deviation.
[2]Data between brackets represent the number in logarithmic cycles reduction.

These facts can be explained considering that the site of action of both hops and PEF is located on the cell membrane (7, 16), improving the cell uptake of hops. In this way, PEF application could allow the reduction of MIC of hops.

Taken together, these results indicate that the antimicrobial agents tested could prevent contamination of the brewing wort if it has to be stored before boiling. In the presence of hops 0.03 mg/ml or higher, PEF application significantly increased its antimicrobial activity. Moreover, as hops and chitosan are thermostable, they can maintain their antibacterial activity even after wort boiling. This provides some protection against bacterial contamination during the brewing process as reported for nisin by Ogden *et al.* (22).

2. Addition of natural antimicrobial compounds during fermentation

As LAB strains are usually present along with yeast cells during the fermentation process, it seemed worthwhile to investigate the inhibitory effect of variable concentrations of nisin, chitosan or hops on a mixed culture of *Pediococcus* sp. and a lager yeast strain inoculated in AME, to test if these compounds could be used to control bacterial contamination during brewing.

Figure 2A shows that in the presence of 0.0002 mg/ml nisin after 2 h incubation, *Pediococcus* sp. viable cells were not detected; comparatively, a reduction of only about 1 log cycle was observed for *S. cerevisiae* strain.

Similar results were obtained in the presence of chitosan. Figure 2B shows a higher resistance to chitosan of yeast cells with respect to *Pediococcus* strain tested, after 2 h incubation. Chitosan at 0.1 mg/ml reduced *Pediococcus* sp. viability significantly (3 log cycles) whereas yeast viability was unaffected. At the highest chitosan concentration tested, no viable bacterial cells were detected and yeast viability decreased by less than 2 log cycles. Moreover, previous results from our laboratory had shown that 0.1 mg/ml chitosan selectively inhibited *Pediococcus* sp. without significantly affecting yeast viability during 100 hours fermentation. Besides, it was found that pH values, ethanol content and main organoleptic properties of beer remained unchanged in the presence of chitosan (6). These results taken together indicate that chitosan can be an important tool to control contamination during brewing. In this context, polycation could be employed as a remedial agent to avoid bacterial spoilage of beer. Moreover, the use of chitosan in a fermenting brew which rapidly decreased pH values (to 4.5 ± 0.1 units) is recommended due to the increase of its antibacterial activity in acidic conditions (6, 12). The same would apply for nisin because an acidic pH positively affects its solubility and antimicrobial activity (32).

In the presence of hop extracts (Figure 2C), a similar selective inhibitory trend was observed. At the highest hop concentration assayed (0.3 mg/ml) no significant decrease in yeast viability was observed but *Pediococcus* viability was reduced approximately by 2 logarithmic cycles. The selection of hop-resistant LAB and yeasts strains along the beer production process can give account of these results as hops is a normal component of beer worts.

The antibacterial effect of PEF was not studied at the brewing stage, as yeast would be more seriously affected than bacterial cells by merely considering the greater size of yeast cells (1, 5, 35).

Our results showed that chitosan (0.1 mg/ml), nisin (0.0002 mg/ml) or hops at typical addition levels allowed in beer (32), would effectively control frequent contaminating genera during the brewing process. Thus, bacterial cells at levels of 10^4 CFU/ml could be controlled without significantly affecting yeast viability. As demonstrated for chitosan (6), the use of nisin seemed not to affect the fermentative capacity of yeast nor the beer flavor as already reported (22).

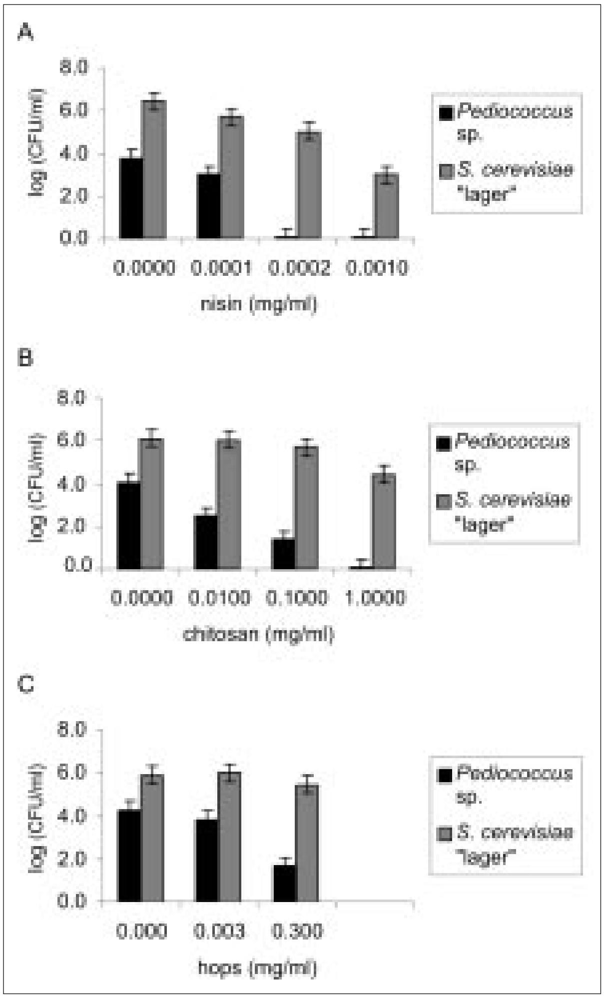

Figure 2. Inhibitory effect of (A) nisin (B) chitosan and (C) hops on the viability of *Pediococcus* sp. and *S. cerevisiae* “lager” in AME after 2 h contact at 4 °C. Data are expressed as mean ± SD (n = 3).

3. Effect of antimicrobial treatments on sporulated bacteria isolated during the bottling process

It has been established that bacterial spores are not only highly resistant to heat but also to electric treatments (30, 36). The effect of nisin, chitosan or hop extracts with and without PEF application on the viability of sporulated cells of *B. megaterium,* previously isolated from the filler system of an industrial brewery, was studied.

A beer containing variable concentrations of added nisin, chitosan or hops and maintained at a pasteurization temperature of 60 °C for 30 min, was inoculated with a *B. megaterium* sporulated culture (@1.10^4 to 5.10^4 CFU/ml). Then, PEF treatment was applied when indicated. Figure 3A shows the effect of variable nisin concentrations with and without PEF application on cell viability. It can be seen that neither nisin nor PEF single treatments were effective. PEF application in the presence of 0.001 mg/ml nisin or higher, increased the inhibitory effect of the electric treatment by an additional reduction of one log cycle. The maximum viability reduction (2 log cycles) was achieved with PEF application in the presence of 0.01 mg/ml nisin. A synergistic effect between nisin and PEF application was observed. A similar result was reported by Pol *et al* (23) on a *B. cereus* laboratory strain subjected to the same treatments in a saline solution and by Terebiznik *et al* for a non sporulated gram negative bacterium in simulated milk ultrafiltrate (33).

Figure 3B suggests that chitosan can be an effective antimicrobial agent against *B. megaterium* cells. The *B. megaterium* viability decreased by more than 2 logarithmic cycles in the presence of 0.01mg/ml chitosan or higher. Application of PEF did not improve bactericidal effect of chitosan.

Hop extract exerted an effective inhibitory effect on *B. megaterium* as can be seen in Figure 3C. Addition of 0.03 mg/ml hops reduced the viability by more than 2 logarithmic cycles with no additional effect of PEF. When PEF was applied in the presence of 0.3 mg/ml hops, no viable cells were detected indicating a synergistic effect under the assayed conditions. As we observed for nisin, these findings are consistent with the notion that hops acts on the cytoplasmic membrane (34).

As nisin and chitosan are heat-stable, they could be added to beer before pasteurization in order to reduce the time and/or temperature of treatment, so as to minimize changes in organoleptic properties and the risk of bottle shattering.

4. Microbiological stability of inoculated beer during storage

Taking into account the previous results, the effect of added nisin (0.0001 mg/ml), chitosan (0.1 mg/ml) or hops

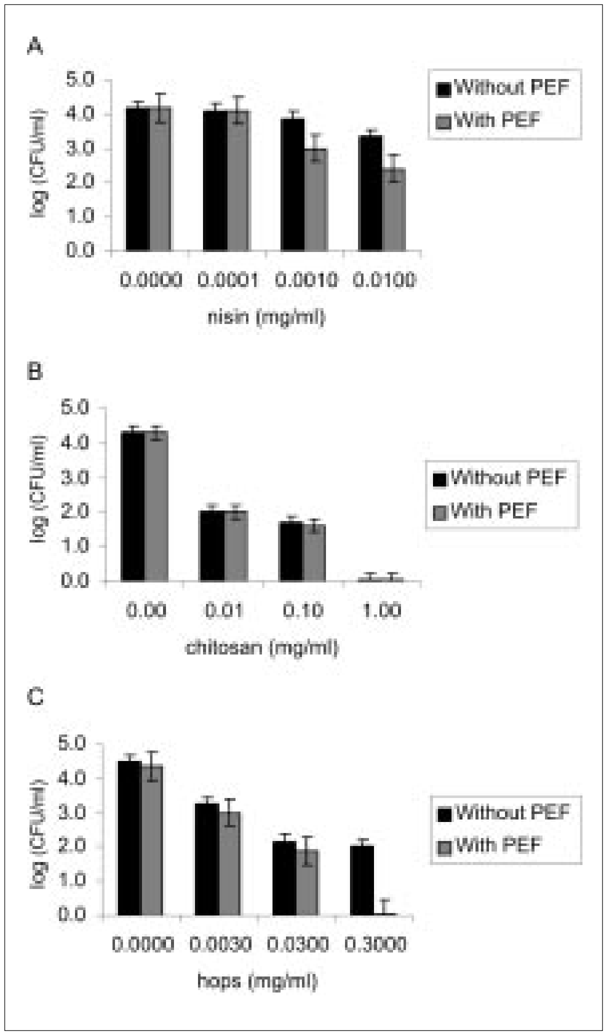

Figure 3. Inhibitory effect of (A) nisin, (B) chitosan and (C) hops on the viability of *B. megaterium* in beer at 60 °C, with or without PEF application

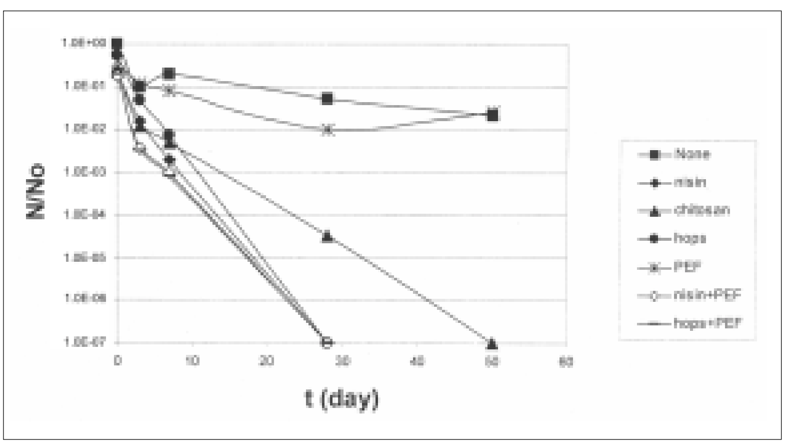

Figure 4. Survival of *Pediococcus* sp. (10^6 CFU/ml) in beer without any treatment (None) and in the beer treated with 0.0001 mg/ml nisin, 0.1 mg/ml chitosan, 0.03 mg/ml hops, PEF, nisin+PEF and hops+ PEF, during 50 days of storage at 25 °C. The results were expressed as the survival fraction (N/No) where N is the number of CFU/ml at 3, 7, 28 or 50 days and No is the initial number of CFU/ml.

(0.03 mg/ml) on the viability of *Pediococcus* sp. (10^6 CFU/ml) in a commercial beer, was studied. The effect of PEF treatment in the presence of nisin and hops was also examined.

Inoculated beer was maintained at 25 °C for up to 50 days. Samples were withdrawn at 3, 7, 28 and 50 days and assayed for viability. As it is shown in Figure 4, the addition of the antimicrobial compounds increased the bactericidal effect on *Pediococcus* sp. in beer. In the presence of hops or nisin, no bacterial viable cells were detected after 28 days storage. Comparable values of cell viability were attained in the presence of chitosan on the 50th day. PEF was applied under mild conditions so that no additional antibacterial effect on bacterial viability compared to control beer was obtained. Nevertheless, PEF treatment increased the antimicrobial effect of nisin or hops. Among the antibacterial treatments used, hops –or nisin– PEF combinations were the most effective in increasing the shelf-life of beer heavily inoculated with *Pediococcus* sp. Similar results were obtained when beer was inoculated with *L. plantarum* isolated from a brewery (data not shown). It is interesting to point out that viability of contaminant bacteria could be reduced by 3 log cycles within 3 days storage when this combination of antimicrobial treatments was applied. Thus, the relatively rapid inactivation of LAB would prevent undesirable changes in beer due to their metabolic activity. Under the experimental conditions, nisin and/or PEF treatments did not alter the physical or organoleptic properties of beer.

PEF application did not improve the antimicrobial effect of chitosan (data not shown). On the other hand, chitosan-treated samples developed a haze that was easily removed from beer by filtration. Thus, this polycation must be used in previous steps of the productive process.

These results suggest that antimicrobial treatments could be used to increase the shelf-life of unpasteurized beers or to complement the traditional pasteurization process. The synergistic antimicrobial effect found between nisin or hops and PEF (see Figure 3A and 3C) opens new possibilities for applying the hurdle concept as a preservation method in beer production (15). On the other hand, the synergistic effect of hops and PEF would only be observed in beers with high content of hops a-isoacids (> 0.016 mg/ml) as in the case of several commercial beers.

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REFERENCES

1. Barsotti L, Cheftel JC. Food Processing by pulsed electric fields. II. Biological aspects. Food Rev Int 1999; 15: 181-213.
2. Daeschel MA. Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technol 1989; 43: 164-7.
3. Dielbandhoesing SK, Zhang H, Caro LHP, van der Vaart JM, Klis FM, Verrips CT, Brul S. Specific cell wall proteins confer resistance to nisin upon yeast cells. Appl Environ Microbiol 1998; 64: 4047-52.
4. Fukao T, Sawada H, Ohta Y. Combined effect of hop resins and sodium hexametaphosphate against certain strains of *Escherichia coli*. J Food Prot 2000; 63: 735-40.
5. Gaskova D, Sigler K, Janderova B, Plasek J. Effect of high-voltage electric pulses on yeast cells: factors influencing the killing efficiency. Bielectrochem Bioenerg 1996; 39: 195-202.
6. Gil G, del Mónaco S, Cerrutti P, Galvagno M. Selective antimicrobial activity of chitosan on beer spoilage bacteria and brewing yeasts. Biotech Lett 2004; 26: 569-74.
7. Hauben KJA, Wuytack EY, Soontjens CCF, Michiels CW. High pressure transient sensitization of *E. coli* to lysozyme and nisin by disruption of outer membrane permeability. J Food Prot 1996; 59: 350-5.
8. Helander IM, von Wright A, Mattila- Sandholm T-M. Potential of lactic acid bacteria and novel antimicrobials against Gram-negative bacteria. Trends Food Sci Technol 1997; 8: 146-50.
9. Hill C. Bacteriocins: natural antimicrobials from microorganisms. En: Gould GW, editor. New methods of food preservation. London, UK, Blackie Academic and Professional, Chapman & Hall, 1995, p. 22-38.
10. Hough JS. Levaduras y bacterias. En: Cambridge University press, Spanish ed. Biotecnología de la cerveza y la malta. Zaragoza, España, Editorial Acribia S.A. 1990, p. 109-29.
11. Jeon Y-J, Park P-J, Kim S. Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr Polym 2001; 44: 71-6.
12. Jumaa M, Furkert FH, Muller BW. A new lipid emulsion formulation with high antimicrobial efficacy using chitosan. Eur J Pharm Biopharm 2002; 53: 115-23.
13. Kalchayanand N, Sikes T, Dunne CP, Ray B. Hydrostatic pressure and electroporation have increased bactericidal efficiency in combination with bacteriocins. Appl Environ Microbiol 1994; 60: 4174-7.
14. Klaenhammer TR. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 1993; 12: 39-86.
15. Leistner L, Gorris LGM. Food preservation by hurdle technology. Trends Food Sci Technol 1995; 6: 41-6.
16. Nikaido H, Vaara M. Molecular basis of bacterial outer membrane permeability. Microbiol Rev 1985; 49: 1-32.
17. No H, Park N, Lee S, Hwang H, Meyers S. Antibacterial activities of chitosan and chitosan oligomers with different molecular weights on spoilage bacteria isolated from tofu. Food Microbiol Safety 2002; 67: 1511-4.
18. No H, Park N, Lee S, Meyers S. Antibacterial activities of chitosan and chitosan oligomers with different molecular weights. Int J Food Microbiol 2002; 74: 65-72.
19. Odgen K. Nisin: A bacteriocin with potential use in brewing. J Inst Brew 1986; 93: 379-83.
20. Odgen K. Cleansing contaminated pitching yeast with nisin. J Inst Brew 1987; 93: 302-7.
21. Odgen K, Tubb RS. Inhibition of beer-spoilage lactic acid bacteria by nisin. J Inst Brew 1985; 91: 390-2.
22. Odgen K, Waites MJ, Hammond JRM. Nisin and brewing. J Inst Brew 1988; 94: 233-8.
23. Pol IE, Mastwijk HC, Bartels PV, Smid EJ. Pulsed electric field treatments enhances the bactericidal action of nisin against *Bacillus cereus*. Appl Environ Microbiol 2000; 66: 428-30.
24. Ralston G, Tracey M, Wrench F. The inhibition of fermentation in baker's yeast by chitosan. Biochim Biophys Acta 1964; 93: 652-5.

25. Roller S, Covill N. The antifungal properties of chitosan in laboratory media and apple juice. Int Food Microbiol 1999; 47: 67-77.
26. Sakamoto K, Konings WN. Beer spoilage bacteria and hop resistance. Int J Food Microbiol 2003; 89: 105-24.
27. Sami MK, Suzuki K, Sakamoto K, Kadokurah H, Kitamoto K, Yoda K. A plasmid pRH45 of *Lactobacillus brevis* confers hop resistance. J Gen Appl Microbiol 1998; 44: 361-3.
28. Schmalreck AF, Teuber M. Structural features determining the antibiotic potencies of natural and synthetic hop bitter resins, their precursors and derivatives. Can J Microbiol 1975; 21: 205-12.
29. Shahidi F, Vidana JK, Jeon YJ. Food applications of chitin and chitosans. Food Sci Technol 1999; 10: 37-51.
30. Simpson MV, Barbosa-Canovas GV, Swanson BG. Combined inhibitory effect of lysozyme and high voltage pulsed electric fields on the growth of *Bacillus subtilis* spores. Annual IFT Meeting, 1995; session 89, paper 2, USA.
31. Strand SB, Nordengen T, Ostgaard K. Efficiency of chitosans applied for flocculation of different bacteria. Water Res 2002; 36: 4745-52.
32. Thomas LV, Clarkson MR, Delves- Broughton J. Nisin. En: Naidu AS editor. Natural Food Antimicrobial Systems. Boca Raton, Florida, CRC Press, 2000, p. 463-524.
33. Terebiznik MR, Jagus RJ, Cerrutti P, Huergo MS de, Pilosof, AMR. Combined effect of nisin and pulsed electric fields on the inactivation of *Escherichia coli*. J Food Prot 2000; 63: 741-6.
34. Ulmer HM, Heinz V, Ganzle MG, Knorr D, Vogel RF. Effects of pulsed electric fields on inactivation and metabolic activity of *Lactobacillus plantarum* in model beer. J Appl Microbiol 2002; 93: 326-35.
35. Wouters P, Bos A, Veckert J. Membrane permeabilization in relation to inactivation kinetics of *Lactobacillus* species due to pulsed electric fields. Appl Environ Microbiol 2002; 67: 3092-101.
36. Yonemoto Y, Yamashita T, Muraji M, Tatbe W, Ooshima H, Kato J, *et al.* Resistance of yeast and bacterial spores to high voltage electric pulses. J Ferment Bioeng 1993; 75: 99-102.

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