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Effect of hippurate additon on benzoate production during lben fermentation

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أثر الإيبرات على تكوين البانزواط في اللبن

استهدف هذا العمل دراسة تأثير إضافة الإيبرات على مقادير البانزواط المكون خلال التخمر وكذلك تأثير البانزواط على نمو الفطريات في اللبن التقليدي. لإظهار تأثير الإيبرات على مقادير البانزواط المكون خلال تخمر اللبن، أخذت عينات من اللبن المضاف وغير المضاف بالإيبرات. وقد تبين أن تكوين حامض البنزويك يتزايد مع كمية الإيبرات المضافة وأن هذا التزايد يتبع ارتدادا خطيا. وكان تحويل حامض الإيبريك إلى حامض البَنْزُويكْ مابين 25-30% في نهاية التخمر الإصطناعي و 40-50% في نهاية التخمر التقليدي. لدراسة تأثير البانزوَاط على نمو الفطريات في اللبن، أخذت عينات من اللبن المضاف بالإيبرات قبل التخمير وأضيفت إليها فطريات ثم تَمَّ تخزينها في الثلاجة لمدة أسبوع. وقد أظهرت النتائج أن كل مقادير البانزواط المكونة من اللبن لن تستطيع إيقاف نمو الفطريات، ولكنها عطلت نموها.

الكلمات المفتاحية : لبن- البانزواط- إيبرات- الفطريات

Effet de l'addition de l'hippurate sur la production de benzoates durant la fermentation du lben

Ont été examinés dans cette étude l'effet de l'addition de l'hippurate sur les niveaux de benzoate durant la fermentation mésophilique et l'effet du benzoate sur la croissance des levures dans le lben traditionnel. La production de l'acide benzoïque dans le lben dépend de façon linéaire de l'ajout de l'hippurate. Le taux de conversion en acide benzoïque varie de 25 à 30% pour le lben industriel et de 40 à 50 % pour le lben traditionnel. Les échantillons de lben inoculés avec une culture de levure et gardés au réfrigérateur pendant 7 jours montrent que le benzoate formé dans le lben n'inhibe pas la croissance des levures, mais augmente la phase de latence. Ces phases ont été estimées à environ 1 jour pour 6,8 mg/kg de benzoate, 1,5 jours pour 14,1 mg/kg, 2 jours pour 30,0 mg/kg et 3 jours pour 46,1 mg/kg.

Mots clés : Lben -Benzoate - Hippurate - Levure - Fermentation lactique

Effect of hippurate additon on benzoate production during lben fermentation

The effect of hippurate addition on benzoate levels produced during mesophilic fermentation and the effect of benzoate on the yeasts growth in traditional lben were examined. To determine the effect of hippurate on benzoate levels produced during lben fermentation process, hippurate added samples and non-added samples were taken at regular intervals. The production of benzoic acid in lben increased as the levels of added hippurate was higher, and this increase followed a linear regression. The conversion of hippuric acid to benzoic acid was between 25 and 30 at the end of fermentation process for industrial lben and between 40 and 50% for traditional lben. To determine the effect of benzoate on yeast growth in lben, lben samples which were added with hippurate were fermented and the lben samples prepared were inoculated with a yeast culture and stored in a refrigerator for 7 days. The results showed that all the benzoate levels produced in lben did not stop yeast growth, but did increase the lag phases. These were estimated to be about 1, 1.5, 2 and 3 days for the respective benzoic acid levels of 6.8, 14.1, 30.0 and 46.1 mg/kg.

Keys words : Lben - Benzoate - Hippurate - Yeast - Milk fermentation

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INTRODUCTION

Benzoates are legal preservatives permitted in many countries and in a variety of food products (Ahlborg *et al.*, 1977). They are used for their antimicrobial activity against yeasts, fungi and some bacteria (Chipley, 1993). The use of benzoic acid is not allowed by most countries in many dairy products (Fondu *et al.*, 1984). In Morocco, benzoates are also not allowed to be used in milk products (MAMVA & MSP, 1997).

One probable explanation is that benzoates are more toxic and less effective than sorbates, which are used extensively in dairy products (Chipley, 1993 ; Branen *et al.*, 1990 ; Puttemans *et al.*, 1985; Sofos & Busta, 1981).

There is no work on the effect of lben fermentation on benzoate production. Milk contains only small amounts of benzoates (Chandan *et al.*, 1977 ; Hatanaka & Kaneda, 1986), but some fermented dairy products can reach higher levels of benzoates (Sieber *et al.*, 1995). Bertling (1985) reported that the presence of benzoic acid in milk products is not due to deliberate addition, but may be the result of unintentional contamination from rennet, veterinary drugs, teat dips, addition of fruit flavorings which contain benzoic acid or bacterial conversion of hippuric acid to benzoic acid. Chandan *et al.* (1977) indicated that benzoic acid is produced by lactic acid bacteria used to prepare cultured dairy products. Nishimoto *et al.* (1969) reported that lactic acid bacteria convert milk hippuric acid to benzoic acid.

In view of the preservative nature of benzoates as well as their implication in technological and legislative aspects of dairy product additives, the present study was undertaken in order to examine the effects of hippurate addition on benzoate levels during mesophilic fermentation and to evaluate the contribution of benzoate to the shelf-life of lben.

MATERIALS AND METHODS

1. Effect of hippurate addition on benzoate production during industrial lben fermentation

This study was conducted in a commercial lben processing site. Lben, a popular milk drink in Morocco was produced from milk containing 1% fat in which the solids content was adjusted by addition of 10% reconstituted nonfat dried milk resulting in a total solids content of about 90 g/l and a fat level of about 5 g/l. The milk is then homogenized, pasteurized (95°C, 5 min) cooled to about 22°C and inoculated with a mesophilic starter culture, supplied by Chr. Hansen's Laboratory, Inc. (Copenhagen, Denmark). Milk samples taken just prior to incubation were added with 0, 10, 20, 40, 60 and 100 mg of sodium hippurate per kg of milk, homogenized and 10.0 ml of each solution were transferred to 24 ml capped test tubes. The tubes were placed in a water bath at 22°C for 48 h.

In order to follow the benzoate levels during lben fermentation process, duplicate sub-samples from each level of hippurate added samples and from non-added samples were taken every 3 h. pH was measured in order to determine the end of the fermentation process, which lasted about 15 h. These samples were refrigerated during 24 h for further benzoic acid determination.

2. Effect of hippurate addition on benzoate production during traditional lben fermentation

The effect of hippurate addition on benzoate production during traditional lben fermentation process was examined. Traditional lben was prepared in the laboratory according to Tantaoui-Elaraki *et al.* (1983). Whole raw milk samples were mixed with 0, 10, 20, 40, 60 and 100 mg/kg of sodium hippurate. They were then homogenized, and left at ambient temperature (20-22°C).

To determine the effect of hippurate addition on benzoate levels during lben fermentation process, duplicate sub-samples from each level of added samples and from non-added samples were taken every 6 h up to 42 h. pH was monitored in order to determine the end of the fermentation process. These samples were used for benzoic acid determinations.

3. Effect of benzoate levels on yeast growth in lben during storage

In order to evaluate benzoate levels on yeast growth, samples which were mixed with 0, 40, 80 and 120 mg/kg of hippurate were fermented in the same way as in the study of the hippurate addition on benzoate levels during traditional lben fermentation outlined before. pH was mesured in order to determine the end of the fermentation process and this was stopped when samples had a pH of 4.6. These lben samples were mixed and 100 ml of each solution were transferred to 200 ml screw capped bottles. They were then inoculated with 1 ml of a dilution of 10^5 CFU/ml of a yeast

culture (*Saccharomyces cerevisiae*). These samples were stored in a refrigerator (6°C) for 7 days, and duplicate sub-samples were taken every day from each level of added hippurate and used for yeasts plate counts. Acidified PDA was used for yeast determination according to APHA (Vauderzant & Splittstoesser, 1992).

Before yeast inoculation and storage, the levels of benzoate were determined in duplicate sub-samples taken from each level of lben added hippurate samples .

4. Benzoate and hippurate determination in commercial milk products

Benzoate concentrations were determined in commercial retail milk and lben products samples. The samples included products from the major processors of milk products in Morocco and were analyzed in duplicate. The numbers of samples were, 6 for liquid milk and 24 for lben.

Benzoate and hippurate concentrations in milk products were determined using a method based on the Provisional Standard of International Dairy Federation (1987), with slight modifications in. For the extraction of benzoate and hippurate from milk products, a sample of 2.0 g was transferred into 10 ml volumetric flask and 2.5 ml of 0.1 M sodium hydroxide solution added to the flask. The sample was mixed, heated in water bath at about 70°C and cooled, the pH was adjusted to 8.0 by adding 0.1 M sulfuric acid solution, then, 2 ml of potassium hexacyanoferrate solution (106 g of potassium hexacyanoferrate in 1 liter of distilled water) and 2 ml of zinc acetate solution (219 g of zinc acetate and 32 ml of acetic acid in 1 liter of distilled water) were added, and finally methanol was used to complete the flask to 10 ml. The solution was transferred to 12 ml centrifuge tube and centrifuged for 10 minutes at 950 g. The supernatant was filtered through a 0.45 mm membrane filter and used for HPLC analysis.

A mobile phase consisting of 10% methanol and 90% phosphate buffer solution (pH 6.7) was maintained at a flow rate of 0.8 ml/min through an endcapped octadecyl column (250x4.6 mm, i.d.) with 5m particle size (IBM instruments Inc., Wallingford, CT, USA).

Benzoic and hippuric acids in milk product extracts were quantified using peak heights from benzoic acid and sodium hippurate standards of known concentrations, using a variable wavelength detector (LINEAR UVIS 204 Detector from Applied Biosystems). The wavelength used was 227 nm.

5. Statistical analysis

Linear regression was used to correlate benzoate production during fermentation, with the level of added hippurate and to determine rate constants for benzoate production during fermentation. An analysis of variance was used to determine the significance of the effect of hippurate addition on benzoate production during fermentation. All statistical analysis used 5% significance. These statistical analyses were carried using STATISTIX software (Analytical software, MN, USA).

RESULTS AND DISCUSSION

1. Benzoate levels in commercial milk products

The number of samples used, the averages and the ranges of benzoic acid levels found in the main moroccan commercial lben brands products are given in Table 1. This table shows that benzoic acid contents of lben products are higher than those of pasteurized commercial milk, which contained only small amounts of benzoic acid. The benzoic acid content in retail Moroccan lben has not been previously reported, but these levels were in agreement with the levels reported by Chandan *et al.* (1977), Geiger (1982), Ito *et al.* (1983), Stijve & Hischenhuber (1984), Sieber *et al.* (1995), Toppino *et al.* (1990), and Drawert & Leupold (1978) in various fermented products. Sieber *et al.* (1995) pointed out in their review that fermented dairy products contained up to 50 mg/kg benzoic acid with most mean values around 20 mg/kg. It was reported that benzoic acid could be considered as natural component of milk products, since it is produced in milk during fermentation from hippuric acid (Sieber *et al.*, 1995 ; Nishimoto *et al.*, 1969).

Table 1. Average benzoic acid levels in milk products

Milk products	Average benzoate in mg/kg	Number of samples	Benzoate range in mg/kg
Milk	2.1	6	2.0-3.1
Lben brand A	5.7	6	5.3-6.0
Lben brand B	11.4	6	9.6-12.0
Lben brand C	17.9	6	16.0-21.0
Lben brand D	16.0	6	14.0-18.0

Brand A,B,C and D : Commercial retail products of main Moroccan processors

2. Effect of hippurate addition on benzoate production in industrial and traditional lben

Benzoic acid production during industrial and traditional lben incubation for all levels of sodium hippurate added are shown in tables 2 and 3 respectively. These tables show also the average pH change of lben samples during fermentation. These were 4.2 and 4.5 after 15 and 36 hours of fermentation in case of industrial and traditional lben, respectively.

The levels of benzoate produced during fermentation increased with time of fermentation. These increase in benzoate levels were better described (higher r^2) by a linear relationship than a semilogrithmic relationship. Regression lines were calculated using 12 data points for each level of added hippurate. The data points at 42 and 48 hours were not used to calculate zero-order rate constants and r^2 for benzoate increase for each level of added hippurate (Table 4).

Table 4. Rate constants and r^2 values for benzoic acid increases during lben incubation for each level of added sodium hippurate

mg/kg of sodium hippurate added	Traditional lben k (mg/kg.hr)	r^2	Industrial lben k (mg/kg.hr)	r^2
0	.12	.99	.16	.97
10	.22	.99	.21	.98
20	.33	.99	.28	.97
40	.44	.98	.45	.98
60	.55	.99	.48	.95
80	.60	.98	.50	.95
100	1.10	.99	.94	.98

Analyses of variance indicated that the levels of hippurate added have a significant effect at the 5% level on benzoate production. The comparison of means, using LSD test showed that all the means are significantly different at 5% level.

The rate constants for each level of hippurate for traditional and industrial lben were not statistically different at 5% level. This means that the rate of benzoate production is the same in

Table 2. Benzoic acid (mg/kg) production during industrial lben incubation for all levels of added sodium hippurate (s.h)

Incubation time in hours	pH of lben	0 mg/kg s.h	10 mg/kg s.h	20 mg/kg s.h	40 mg/kg s.h	60 mg/kg s.h	80mg/kg s.h	100 mg/kg s.h
3	5.6	1.4	1.6	2.6	3.1	5.7	7.7	5.0
6	4.8	1.6	1.8	2.9	3.3	6.1	8.4	10.8
9	4.6	1.8	2.3	3.7	4.8	7.1	10.2	12.0
12	4.4	2.5	3.2	5.2	7.3	9.6	11.6	13.7
15	4.2	3.3	4.0	5.6	7.8	10.9	13.3	17.6
48	3.8	3.5	4.8	6.6	8.6	11.4	14.2	18.1

each value of benzoic acid is an average of duplicate samples.
*average pH of samples with all added levels of sodium hippurate.

Table 3. Benzoic acid (mg/kg) production during traditional lben incubation for all levels of added sodium hippurate(S.h)

IIncubation time in hours	pH*of lben	0 mg/kg s.h	10 mg/kg s.h	20 mg/kg s.h	40 mg/kg s.h	60 mg/kg s.h	80 mg/kg s.h	100 mg/kg s.h
6	6.7	1.3	1.6	2.2	4.0	9.5	10.6	11.2
12	6.5	2.3	2.7	4.4	6.3	12.1	15.1	16.7
18	6.2	3.1	3.3	5.3	8.3	12.3	16.9	18.6
24	5.4	3.8	4.2	7.7	14.4	15.9	23.8	30.4
30	4.9	4.3	6.7	8.7	16.1	22.2	24.8	32.2
36	4.5	5.7	7.7	12.6	17.2	23.7	29.0	43.0
42	4.0	3.5	5.9	8.0	16.6	20.8	22.7	27.5

each value of benzoic acid is an average of duplicate samples
*average pH of all levels of added sodium hippurate.

traditional and industrial lben and depend only on hippurate level added.

The production of benzoic acid at the end of fermentation of industrial lben (15 h) and traditional lben (36 h) increased as the levels of added hippurate increased. These increase followed a linear regression with r^2 of 0.98 and 0.96 for traditional and industrial lben, respectively.

The slope of the line for traditional lben was about twice the slope in case of industrial lben. This means that the conversion of hippurate to benzoate in case of traditional lben was about twice that of industrial lben.

These conversions at the end of fermentation were between 25 and 30% for industrial lben and between 40 and 50% for traditional lben. These conversion were calculated taking into account the average natural content of hippuric acid in milk samples used in this study, which was 20 mg/kg.

These results confirm that benzoic acid is produced naturally during fermentation from hippurate as was reported by various authors (Chandan *et al.*, 1977 ; Sieber *et al.*, 1995 ; Nishimoto *et al.*, 1969 ; Nishimoto *et al.*, 1968).

Industrial lben was made with a mesophilic culture containing *Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*.

Tantaoui-Elaraki *et al.* (1983) reported that the traditional lben fermentation was due mainly to streptococci and leuconostocs, but lactobacilli, yeasts and molds were also present.

Dellaglio (1988) reported that streptococci hydrolyse hippurate weakly. Therefore, there are may be the main producer of benzoate from hippurate during lben fermentation.

The benzoate levels showed a slight decrease in traditional lben after 42 hours, but this did not happen during industrial fermentation after 48 hours. This difference is may be related to the difference of the microrganisms present in the two products.

3. Effect of benzoate levels on yeast growth in lben during storage

The variation of yeast counts in traditional lben samples with time of storage and benzoates levels produced during fermentation are given in Table 5.

These data confirms the results given before, that the production of benzoate in lben depends of the amount of hippurate in milk. The results show that all the benzoate levels produced in lben did not stop yeast growth, but did delay the growth by increasing the lag phase. These were about 1, 1.5, 2 and 3 days for the respective benzoic acid levels of 6.8, 14.1, 30.0 and 46.1 mg/kg.

Table 5. Benzoate levels effects on yeast counts during storage at 6°C for 7 days of traditional lben samples

Hippurate added (mg/kg)	Benzoic acid (mg/kg)*	Lben log yeast counts with days of storage						
		1	2	3	4	5	6	7
0	6.8	4.3	6.0	6.2	8.0	9.0	10.0	10.2
40	14.1	4.3	4.5	6.0	7.0	8.2	8.6	9.7
80	30.0	4.3	4.3	5.0	6.3	7.8	8.2	9.0
100	46.1	4.3	4.3	4.3	5.5	6.5	7.0	7.8

* : Benzoic acid levels obtained at the end of fermentation.

These finding are in agreement with those of Suriyarachchi & Fleet (1981) , who found that the growth of yeasts in yogurt was related to the ability of the yeasts to grow at refrigeration temperature and in the presence of high levels of benzoates. Osborne & Pritchard (1974) reported also that the addition of 150 ppm sodium benzoate to yoghurt did not prevent yeasts proliferation.

REFERENCES

Ahlborg U.G., Dick J. & Eriksson H.B. (1977) Data on preservatives in food. *Var Foeda* 29 (2) : 41-96

Bertling L.K. (1985) Free of preservatives but still positive for benzoic acid? *Deutsche-Milchwirtschaft* 36(5) : 135-136

Branen A.L., Davidson P.M. & Salminen S. (1990) Food Additives Ed. Marcel Dekker Inc. New York.

Chandan R.C., Gordon J.F. & Morrisson A. (1977) Natural benzoate content of dairy products. *Milchwissenschaft* 32(9) : 534-537

Chipley J.R. (1993) Sodium benzoate and benzoic acid *In* Antimicrobials in Foods, pp.11-35, 2nd Edition, ed. P.M. Davidson & A.L. Branen. Marcel Dekker, New York

Dellaglio F. (1988) Chap. II : Starters for fermented milks. Section 1 : Taxonomy and metabolism. *In* Fermented Milks : Science and Technology. *Bulletin of The IDF* 227 : 19-26

Drawert F. & Leupold G. (1978) Uber das Vorkommen von Benzoesaure in Milch und Milcherzeugnissen. *Lebensmittelchem. Gerichtl. Chem.* 32(4) : 76-77

Fondu M., Van Gindertael-Zegers, De Beyl H., Bronkers G., Stein A. & Carton P. (1984) Food Additives Tables updated edition. Ed. Elsevier, Amsterdam

Geiger U. (1982) Benzoesaure in Sauer-milcherzeugnissen und Frischkase. HPLC-Analyse von Benzoesaure, Sorbinsaure und PHB-Estern in Lebensmitteln. *Lebensmittelchem. gerichtl. Chem.* 36 : 109-110

Hatanaka H. & Kaneda Y. (1986) Rapid and simultaneous analysis of hippuric and benzoic acids in fermented milk or raw milk by HPLC. *J. Food Hyg. Soc. Japan* 27(1) : 81-86

International Dairy Federation (1987) Determination of benzoic and sorbic acid content in milk, dried milk, yogurt and other fermented milks. *International Provisional Standard*, 139

Ito Y., Tonogai Y., Ogawa S., Yokoyama T., Honda T. & Iwaida M. (1983) Rapid simultaneous determination of benzoate and sorbate in yoghurt by use of high-performance liquid chromatography. *Japanese J. Dairy Food Sci.* 32(3) : A89-A94

MAMVA & MSP (1997) Circulaire conjointe, N° 001/97 du Ministère de l'Agriculture et de la Mise en Valeur Agricole et du Ministère de la Santé Publique relative à l'utilisation des additifs alimentaires.

Nishimoto T., Uyeta M. & Taue S. (1969) Precursor of benzoic acid in fermented milk. *J. Food Hyg. Soc. Japan* 10 : 410-413

Nishimoto T., Uyeta M., Taue S. & Takebayashi I. (1968) Bacterial formation of benzoic acid in milk. *J. Food Hyg. Soc. Japan* 9 : 58-59

Osborne R.J.W. & Pritchard E.W. (1974) Preservation of fruit yoghurt by preservatives and by storage at low temperatures. *XIX International Dairy Congress, 1E* 809-810

Puttemans M.L., Branders C., Dryon L. & Massart D.L. (1985) Extraction of organic acids by ion-pair formation with tri-n-octylamine. VI : Determination of sorbic acid, benzoic acid and saccharin in yogurt. *J. Assoc. Off. Anal. Chem.* 68 : 80-82

Sieber R., Butikofer U., Baumann E & Bosset J.O. (1995) Benzoic acid as a natural compound in cultured dairy products and cheese. *Int. Dairy J.* 5 : 227-246

Sofos J. N. & Busta F.F. (1981) Antimicrobial Activity of sorbates. *J. Food Prot.* 44(8) : 614-622

Stijve T. & Hischenhuber C. (1984) High performance liquid chromatographic determination of low levels of benzoic acid and sorbic acid in yoghurt. *Dtsch. Lebensm. Rundsch.* 80 : 81-84

Suriyarachchi V. R. & Fleet G. H. (1981) Occurence and growth of yeasts in yogurt. *App. Environ. Microbiol.* 42(4) : 574-579

Tantaoui-Elaraki A., Berrada M., El Marrakchi A. & Berramou A. (1983) Étude sur le lben marocain. *Le lait* 63 : 230-245

Toppino P.M., Volpato R., Amelotti G. & Contarini G. (1990) Determinazione quantitativa HPLC di acido ascorbico e acido benzoico nei prodotti latierro-casaeri. *Sci. Technol. Latt. Cas.* 41 : 137-152

Vauderzant C. & Splittstoesser D.F. (1992) Compendium of Methods for the microbiological examination of foods 3rd Edition Am. Publ. Health Assoc. Washington DC., USA.