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HAEMOLYTIC ACTIVITY OF *Enterococcus faecium* ISOLATED FROM DIFFERENT EGYPTIAN DAIRY PRODUCTS

El Attar, Aisha
Dairy Science Depart. Fac. of Agric. Alex. Univ., Alex., Egypt.

ABSTRACT

Using of strains belonging to the genus *Enterococcus* sp. as starter is still an intersection in food safety. Therefore, it is very important to examine the pathogenicity of starter cultures by the hemolytic activity. One hundred strains of *E. faecium* were isolated from different Egyptian dairy products which screened for hemolytic activity. Subsequently, the aminopeptidase and autolytic activities of non hemolytic strains were studied as these temperaments play a major role in cheese ripening and flavour development.

Most of these tested strains (82 %) were of non hemolytic reaction (γ-hemolysis), while there was only one positive hemolytic strain (β-hemolysis) and the remaining strains had a partial hemolytic reaction (α- hemolysis). Fifty percent of non hemolytic tested strains were examined for their aminopeptidase and autolytic activities. The lowest value of aminopeptidase activity was noticed in the majority of these tested strains (51.2 %). Only 7.3 % of strains had a high value of this activity. On the other hand, 46% of these tested strains had a moderate autolytic activity, followed by 39 % and 15 % which had minimal and high aptitude of autolytic activity, respectively. It is very important not only to fulfill the required qualification of such strains as aromatic producer, but also as a safer tool implements in our foods.

Keywords: Hemolysis, *Enterococcus faecium,* Aminopeptidase and autolytic activities, Egyptian dairy products

INTRODUCTION

Enterococci are widely distributed in nature and they gain entry into milk and milk products. The natural habitat of these organisms is the intestinal tract of humans and animals. Occasionally, they can establish themselves in an epiphytic relationship on growing vegetation and thus can also grow outside their natural habitat. So, the presence of enterococci in many dairy products makes it difficult to interpret the significance of their presence, since their origin is intestinal and they are considered as indicators of fecal contamination because of their possible association with other enteric pathogens. An European Economic Community directive (EEC, 1992) gives the health regulations for milk-based products and does not set a limit for the microbial concentration of enterococci. Several authors were prompted to consider some species of the genus *Enterococcus* as ordinary components of the lactic microflora of cheese, not as indicators of fecal contamination, as far as these products are concerned (Gatti *et al.,* 1993 and 1994). Despite this, some food hygienists are still reluctant to accept high levels of enterococci in cheese, primarily because of some biochemical activities associated with the possible pathogenicity. Hemolytic activity is known to be associated with the virulence of enterococci (Moellering, 1992). Generally, hemolysis is used in the empirical identification of microorganisms based on the ability of bacterial colonies grown on agar plates to break down red blood cells in the culture. When the organism has been grown on blood agar plates, it can be classified

with regard to whether or not it has caused hemolysis in the red blood cells (RBC_s) incorporated in the medium. A substance that causes hemolysis is a *hemolysin*. When the tested strains are grown on blood agar, their colonies are surrounded by a yellowish halo of complete clearing on a background of the bright red agar, called beta hemolysis (β-hemolysis). Some strains produce partial hemolysis on blood agar, and produce turbid halos with a greenish cast around the colonies, termed alpha hemolysis (α- hemolysis). Those strains which produce no lysis are termed gamma hemolysis (γ-hemolysis).

In order to evaluate the potential risk of *Enterococcus faecium* occurring in some Egyptian dairy products and their possible use as starters for cheese production, this work was undertaken to characterize a large number of *E. feacium* strains of dairy origin with the aim of ruling out their possible pathogenicity by studying their hemolytic activities. Consequently, studying the aminopepptidase and autolytic activities for non hemolytic tested strains to be applied in the proceeding of dairy products.

MATERIALS AND METHODS

Bacterial strains and growth conditions

One hundred strains of *Enterococcus feacium* were isolated from different dairy products (Milk, fermented milk and cheese) and were identified in the laboratory of the biochemistry of dairy microorganisms, Department of Dairy Science and Technology at the Faculty of Agriculture, Alexandria University, as mentioned by El Attar *et al* 2002. Identified strains were cultivated in M17 medium at 42ºC.

Haemolytic activity

The hemolytic activity was done by streaking the tested cultures in duplicate which were reactivated in M17 broth and inoculated on blood agar base (Biolife S.r.I, Milano. Italy) which supplemented with 5% defibrined human blood (El Shattpy hospital for children, Alexandria). After incubation at 37ºC/24h, the hemolysed zones around the colonies were evaluated. These were classified as β, α or γ, according to the appearance of clear hemolysed zones, small greenish or, non hemolysed area of agar under and around the colony, respectively.

The separation of biomass

In order for the dairy industry to consider any culture as a starter culture, the candidate culture has to fulfill a number of criteria. Economical aspects such as the propagation must be economically feasible with a high yield of biomass; the produced cells should be easily separated by the centrifugation or the microfiltration processes (Buckenhuskes, 1993).

The separation of biomass was accomplished as mentioned by Ayad *et al* 2004. The optical density of the obtained supernatant that resulted in after the centrifugation was measured at 650nm (OD_{650}) and used to express the biomass separation. A zero reading was taken as an indication for excellent separation and the OD_{650} ranged from 0 to 0.1 indicated a good separation of biomass, while OD_{650} ranged from 0.2 to 0.3 and more than 0.3 indicated a fair and poor biomass separation, respectively. In this

study, only strains having excellent and good biomass separations were selected for determination the amiopeptidase and autolytic activies.

Determination of Aminopeptidase activity

Cells were harvested at early stationary phase by centrifugation at 10000 xg for 20 min at 4ºC. The pellets were then washed twice with 0.01M potassium phosphate buffer pH 7.0 and stored at -20°C. The method of Miozzari *et al.,* (1978) was used for the permeabilization to evaluate the proteolytic activity in the previous obtained pellets.

The substrate used for the determination of aminopeptidase activity was L-leucyl paranitroanilide (Leu-*p*NA) as described in the method of El Soda and Desmazeaud (1982). One unit of enzymatic activity was defined as the amount of enzyme producing a change of 0.01 unit/min of absorbance at A_{410}.

Measurement of the rate of autolysis

A portion of cell suspension was added to 0.01M phosphate buffer pH 5.5 containing 1M sodium chloride to obtain an optical density of 0.9-1.0 at 650nm and incubated at 37ºC. After different time intervals, the percentage decrease in Optical Density was measured and expressed as % autolysis (Thiboutot *et al.,* 1995).

RESULTS AND DISCUSSION

Haemolytic activity

The hemolytic activity of 100 tested strains of *E. faecium* was reported in Figure (1). The γ-hemolysis reaction was exhibited by 82% of these tested strains, and only few strains (17%) were α-hemolysin producer. On the other hand, one strain (1%) had the ability to produce hemolysin (β-hemolysis). The low percentage of *E. faecium* strains which produced β-hemolysis was noticed previously by Trovatelli *et al.* 1987 and Giraffa *et al*. 1995. So, the β-hemolytic enterococci include these strains which could be pathogenic to both humans and animals. For this reason, this work was focused on the positive hemolytic activity of dairy enterococci on blood agar which, however, appears to be only a rough guide to pathogenicity (Collins *et al.* 1989)

Aminopeptidase and autolytic activities

The proteolytic activity of dairy Lactic Acid Bacteria (LAB) is essential for the bacterial growth in milk and it is involved in the development of organoleptic properties of different fermented milk products (Axelsson, 1998; Christensen *et al.,* 1999). The aminopeptidase activity (AP) was determined to express the proteolytic activity of strains. Aminopeptidase and autolytic (AU) activities of 50% of non hemolytic tested strains were illustrated in Table (1). These strains have been selected according to the separation of biomass. The high aminopeptidase and autolytic activities were observed for 7.3% and 15% of strains respectively. There was only one strain recorded high values of both AP and AU activities, while, both moderate AP and AU activities were observed in 6 strains. Therefore, the ability of strains to lyse and subsequent release of their intracellular enzymes is a desirable trait during the ripening of cheese, the degree of autolysis is strain dependent (Wilkinson *et al.,* 1994; El-Soda *et al.,* 2000). Results in Table (1) revealed that the majority of non hemolytic tested strains of *E. faecium* (46%) exhibited

a moderate autolytic activity. Whereas, the lowest values of AP activity was corresponded to the preponderance of these tested strains (51.2%). The studying of aminopeptidase and autolytic activities in non hemolytic *E. faecium* can be applied in the manufacture of different dairy products. So, this study was to elucidate the role that enterococci play in cheese ripening. However, as enterococci have been recognized in recent years as major nosocomial pathogens, one should carefully consider the potential virulence factors of this group of microorganisms before use. The British "Advisory Committee on Novel Foods and Processes" (ACNFP) accepted the use of *E. faecium* strain K77D as a starter culture in fermented dairy products (ACNFP, 1996). Also, It has been proposed that enterococci can be included as part of defined starter cultures for different European cheeses. The effect of enterococci as starter cultures or co-cultures was studied in cheeses, such as Feta cheese, strains belonging to the species of *E. faecium* (Sarantinopoulos et al., 2002) have been tested.

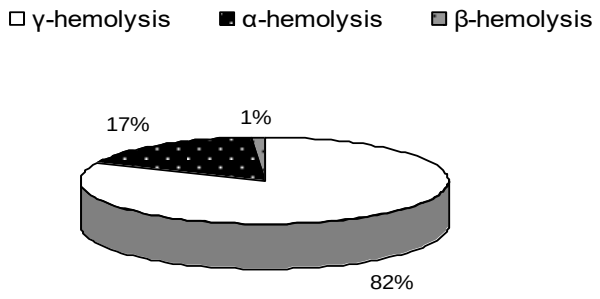

Figure1: Hemolytic activity of *E. faecium* cultivated at 37°C for 24 hrs

Table 1: The percentage of non hemolytic tested *E. faecium* producing aminopeptidase and autolytic activites

Aminopeptidase activity[a]			Autolytic activity[b]		
High	Moderate	Poor	High	Moderate	Poor
%۷،۳	41.5%	51.2%	15%	46%	۳۹%

[a] **Aminopeptidase activity level: high, 13–19 U/OD_{650}; moderate, 6–13 U/OD_{650}; poor, 0.8–5 U/OD_{650}.**

[b] **Autolytic activity level: high, 35–66%; moderate, 24–34%; poor, 0–23%.**

REFRENCES

ACNFP, 1996. Report on Enterococcus faecium, strain K77D. MAFF Advisory Committee on Novel Foods and Processes, Report, Ergon House c/o Nobel House, 17 Smith Square, London SW1 3JR, United Kingdom.

Axelsson, L., 1998. Lactic acid bacteria: classification and physiology. In: Salminen, S., von Wright, A. (Eds.), Lactic Acid Bacteria: Microbiology and Functional Aspects. Marcel Dekker, New York, pp. 1–72.

Ayad, E.H.E., Nashat, S., El-Sadek. N., Metwaly, H., El-Soda M. (2004) Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological criteria. Food Microbiology. 21, 715–725

Buckenhuskes, H. J. (1993) Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiol. Rev. 12, 253-272.

Christensen, J.E., Dudley, E.G., Pederson, J.A., Steele, L.J., 1999. Peptidases and amino acid catabolism in lactic acid bacteria. Antonie van Leeuwenhoek 76, 217–246.

Collins, C.H., Lyne, P.M., and Grange. J.M. (1989). Collins and Lyne's Microbiological Methods. 6th ed. Butterworths and Co., London, England

EEC 1992. Consigilo delle Comunità Europee Direttiva 92/46/CEE del 16.06. 1992. Gazz. Uff. delle Comunità Europee N. L 268/1. September 14. 1982.

El Attar. A., Awad. S. and El Soda. M. (2002). Antibiotic resistance among *Enterococcus faecium* isolated from different dairy products. Proceddings of the 2nd Congress on Recent Technologies in Agriculture, Faculty of Agriculture, Cairo University, 28-30 October. (.3). 600.

El-Soda, M., and Desmazeaud, M. (1982). Les peptides –hydrolase des Lactobacillus du groupe Thermobacterium. 1. Mise en evidence de ces activités chez *Lactobacillus helveticus*, *L. acidophilus*, *L. lactis* et *L. bulgaricus*. Can. J. Microbiol. 28, 1181-1188

El-Soda, M., Madkor, S.A., Tong, P.S., 2000. Evaluation of commercial adjuncts for use in cheese ripening: 4. Comparison between attenuated and not attenuated lactobacilli. Milchwissenschaft

55 (5), 260–263.

Gatti. M., Fornasari. E. and Neviani. E. (1993). Enterococci nei fromaggi italiani. Latt. (4). 392.

Gatti. M., Fornasari. E., Carminati. D., Giraffa. G. and Neviani. E. (1994). Gli enterococci nei fromaggi italiani: attività biochimiche e significato tecnologico. Ind. Latte. (30).11.

Giraffa.G., Pepe. G., Locci. F., Neviani. E. and Carminati. D. (1995). Hemolytic activity, production of thermonuclease and biogenic amines by dairy enterococci. Italian Journal of Food Science. (4), 341-349.

Miozzari, G.F., Nuederberger. P., and Hütter, R. (1978), Analytical Biochemistry, 90, 220-233.

Moellering, R.G. Jr. 1992. Emergence of *Enterococcus* as a significant pathogen. Clin. Infect. Dis. 14, 1173.

Sarantinopoulos, P., Kalantzopoulos, G., Tsakalidou, E., 2002. Effect of Enterococcus faecium on microbiological, physicochemical and sensory characteristics of Greek Feta cheese. International Journal of Food Microbiology 76, 93– 105.

Thiboutot, H., Dako, E., El Soda, M., Vuillemard., .J.C., Power N. and. Simard, R. E (1995) Influence of heat and freeze shocking on the autolysis and peptidase activities of *Lactobacillus casei*. Milchwissenschaft 50 (8) 448-452.

Trovatelli. L., Schiesser. A. and Massa. S. (1987). Identification and significance of enterococci in hard cheese from cow and sheep milk. Milchwissenschaft. (42). 717

Wilkinson, M.G., Guinee, T.P., O'Callaghan, D.M., Fox, P.F., 1994. Autolysis and proteolysis in different strains of starter bacteria during cheese ripening. J. Dairy Res. 61, 249–262.

النشاط التحللي للدم لبكتريا *Enterococcus faecium* المعزولة من مختلف المنتجات الألبان المصرية

عيشة العطار

قسم علوم و تكنولوجيا الألبان. كلية الزراعة. جامعة الأسكندرية. الأسكندرية. ج.م.ع

ما زال استخدام البادئات التابعة لجنس ال *Enterococcus* مثار للجدل من الناحية الأمنة للغذاء. و لذا فمن الضروري اختبار تحلل الدم للمزارع البكترية التابعة لجنس ال *Enterococcus* لمعرفة مدي تأثيرها علي صحة مستهلكي منتجات الألبان.

تم عزل مائة سلالة من ال *Enterococcus faecium* من منتجات البان مصرية مختلفة و التي تم دراسة النشاط التحللي للدم لها. و تبعا لذلك تمت دراسة كل من أنشطة الأمينوبيتيداز و التحلل الذاتي للسلالات الغير محللة للدم. حيث أن هذه الأنشطة تلعب دور هام في تسوية الجبن و تطور النكهة بها.

معظم السلالات المختبرة (٨٢٪) كانت غير محللة للدم من النوع جاما, بينما كانت هناك سلالة واحدة فقط محللة للدم من النوع بيتا وباقي السلالات كان لها نشاط تحللي جزئي للدم من النوع ألفا.

تمت دراسة نشاط الأمينو ببتيداز و نشاط التحلل الذاتي ل ٥٠٪ من السلالات الغير محللة للدم. و قد لوحظ أنخفاض قيم نشاط الأمينو ببتيداز لمعظم هذه السلالات (٥١,٢٪). فقط ٧,٣٪ من هذه السلالات لها قيم عالية عند دراسة هذا النشاط.

من جهة اخري ٤٦٪ من هذه السلالات كان لها نشاط تحللي ذاتي متوسط و يليها ٣٩٪ و ١٥٪ من السلالات كانت ذات نشاط منخفض و مرتفع علي التوالي.

و لذا فمن الضروري ليس فقط تحقيق المواصفات المطلوبة لكل السلالات من حيث انتاجيتها لمواد الطعم و لكن ايضا التأكد من مدي سلامة تواجدها في غذائنا.

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