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Note

Incidence of virulence in bacteriocin-producing enterococcal isolates

Hittu GUPTA, Ravinder Kumar MALIK*

National Dairy Research Institute, Karnal, Haryana, India

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Abstract – Enterococci isolated from a total number of 68 dairy products and 28 human fecal samples were screened for bacteriocin production. The incidence of the bacteriocin-production trait was highest among the cream samples (33.51%), followed by *dahi* samples (15.55%), raw milk samples (9.51%), Cheddar cheese (9.2%) samples and human feces (4.72%). The isolates showed antilisterial effect besides activity against other lactic acid bacteria. Sixty potent bacteriocin-producing isolates of enterococci were evaluated for the incidence of virulence characters for their safe exploitation. None of the isolates was found to exhibit gelatinase, DNAse or β-hemolysis activity. Furthermore, only 4 bacteriocin-producing isolates among 60 were observed to be vancomycin-resistant. It was concluded that bacteriocin-producing *Enterococcus* strains lacking hemolytic activity and not carrying vancomycin resistance genes may be safely exploited as starter cultures, co-cultures or probiotics.

bacteriocin / *Enterococcus* / enterocin / vancomycin / starter culture

摘要 – 产细菌素肠球菌的分离及其毒性。从 68 份乳制品和 28 份人的粪便样品中分离出来的肠球菌经检测能够产生细菌素。在所有样品中分离出的能够产生细菌素的肠球菌菌株最多的是奶油 (33.51%), 其次是达希酸奶 (15.55%) 、原料奶 (9.51%) 、契达干酪 (9.2%)、人的粪便 (4.72%) 。分离出的肠球菌除了具有抗乳酸菌的活性外, 还具有抗李斯特菌的活性。基于对新开发微生物菌株的安全性考虑, 对 60 株分离出来的具有潜在抗菌素活性的肠球菌的毒性进行了评价。实验证明, 没有一株肠球菌出现白明胶酶、脱氧核糖核酸酶和 β-溶血作用现象。此外, 在 60 株分离的肠球菌中只发现了4 株耐万古霉素的肠球菌。可以认为, 产生细菌素的肠球菌缺少溶血活性因而不能携带耐万古霉素基因, 因此可以将这些肠球菌开发成为发酵剂、共发酵剂或益生菌。

抗菌素 / 肠球菌 / 肠球菌素 / 万古霉素 / 发酵剂

Résumé – Incidence de la virulence d'isolats d'entérocoques produisant une bactériocine. Des entérocoques isolés de 68 produits laitiers et 28 échantillons fécaux humains ont été criblés pour leur production de bactériocine. L'incidence de ce caractère était maximale dans les échantillons de crème (33,51 %), suivis des échantillons de *dahi* (15,55 %), de lait cru (9,51 %), fèces humains (4,72 %) et fromage Cheddar (9,2 %). Outre leur activité contre les autres bactéries lactiques, les isolats avaient un effet anti-listeria. Soixante isolats d'entérocoques ayant la capacité de production de bactériocine ont été évalués pour l'incidence de leurs caractères de virulence sur leur sécurité d'emploi. Aucun des isolats n'a montré d'activité gélatinase, DNAse et β-hémolyse.

* Corresponding author (通讯作者): rkm.micro@gmail.com

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De plus, seuls 4 d'entre eux se sont avérés résistants à la vancomycine. Il a été conclu que les souches d'*Enterococcus* produisant une bactériocine, qui n'ont pas d'activité hémolytique et ne portent pas de gènes de résistance à la vancomycine, présentent une sécurité suffisante pour leur emploi dans des levains, cocultures ou probiotiques.

bactériocine / *Enterococcus* / entérocine / vancomycine / levain

1. INTRODUCTION

Studies on bacteriocins produced by lactic acid bacteria (LAB) have attracted increasing interest in recent years because of their potential use as biopreservatives in the food industry to eliminate spoilage and food-borne pathogenic bacteria [36]. These bacteriocins fulfill all the requirements of an ideal food preservative without showing any deleterious effect on the food. Furthermore, they are digested by human proteolytic enzymes in the gastrointestinal (GI) tract. Bacteriocins produced by LAB are ribosomally synthesized bactericidal peptides or proteins that are cationic, hydrophobic or amphiphilic molecules of 20 to 60 amino acid residues [33, 47].

Enterococci are Gram-positive bacteria, which fit the general definition of LAB. They are often isolated from dairy systems as desirable microbiota and are believed to play an important role in cheese ripening due to their high lipolytic, proteolytic and flavor-producing activities [21, 28]. Investigations have resulted in the recognition that production of antilisterial bacteriocins is a common characteristic within this group of bacteria [3, 21, 37, 54]. Enterocins, the antibacterial peptides produced by *Enterococcus* species are small, hydrophobic and thermostable with activity over a wide range of pH. Enterocins are active against many Gram-positive bacteria encompassing undesirable and pathogenic microbes, such as *Clostridia, Bacillus, Staphylococcus* and *Listeria,* as well as some Gram-negative bacteria [22, 23, 43]. There are several reports regarding the application of bacteriocin-producing enterococci as starter or protective cultures or use of their bacteriocins for protection against spoilage and pathogenic bacteria in cheese [21, 28, 30, 50]. This fact stimulates the special interest in enterocins as potential food preservatives. Furthermore, enterococci are used as probiotics in some countries [31]. The strain *Ent. faecium* SF68 has been studied in detail as a probiotic in the treatment of antibiotic-associated diarrhea [23, 40].

Some potential drawbacks of the genus *Enterococcus* have often been the subject of controversy. In the recent reviews on the potential risks related to the use of enterococci in food technology it has been reported that some *Ent. faecalis* strains are associated with a number of human infections *viz.,* endocarditis, urinary tract infections and bacteraemia, and a few *Ent. faecium* strains have also been implicated as the causative agent in enterococcal infections [8, 21, 25, 53]. Furthermore, in recent years, there has been an emergence of antibiotic-resistant enterococci (ARE), *Ent. faecium* being the predominant enterococcal species in the subset of vancomycin-resistant (VRE) isolates [45]. However, there exists strong phenotypic variability within the genus *Enterococcus* and some significant properties, such as antibiotic resistance and potential pathogenicity, have been reported to be dependent on the source of the isolate [23, 25, 38].

Since bacteriocin producers are being exploited in the food industry for the purpose of biopreservation in place of chemical preservatives, the safety of such strains is of major concern. The present investigation was carried out to study the frequency of enterococci and bacteriocin producing

enterococci in dairy products. Furthermore, a study regarding the incidence of virulent characters in the bacteriocinogenic enterococci was also carried out to investigate the safety of such strains for their safe application in food products.

2. MATERIALS AND METHODS

2.1. Bacterial strains

A total number of 68 samples of dairy products comprising raw milk (42), raw cream (13), *dahi* (7) and Cheddar cheese (6) was obtained from various sources such as the local market and the Dairy of the National Institute for the isolation of enterococci. Enterococci were also isolated from 28 human fecal samples. Decimal dilutions of the samples were plated on Citrate Azide Agar (CAA) [51] and incubated at 37 °C for 3 days. A total number of 1061 isolates was randomly selected. After microscopic examination, the enterococcal isolates, sub-cultured to purity on MRS medium [13], were tested for Gram stain and catalase reaction. Isolated colonies were characterized to genus level by physiological, biochemical and morphological tests according to the criteria suggested by Facklam and Collins [19] and Devriese et al. [16]. They were then frozen at –80 °C in MRS broth containing 50% glycerol.

2.2. Bacterial cultures, media and culture conditions

Strains of *Micrococcus luteus, Enterococcus faecalis, Lactococcus lactis* subsp. *cremoris, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei* subsp. *casei, Lactobacillus brevis, Pseudomonas aeruginosa, Staphylococcus aureus, Shigella dysenteriae, Salmonella typhi* and *Escherichia coli* were obtained from the National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal, India. Indicator strains of *Pediococcus acidilactici* LB 42 and *Listeria monocytogenes* Scott A were kindly provided by Prof. Bibek Ray (Department of Animal Science, University of Wyoming, Laramie, Wyoming, USA) and culture of *Enterococcus faecalis* DSM 20478 (Tab. I) was kindly provided by Dr. Ulrich Schillinger (Institute of Hygiene and Toxicology, Federal Research Center for Nutrition, Karlsruhe, Germany).

Lactobacilli, pediococci and enterococci were propagated in MRS broth [13]; *L. lactis*, *Strep. thermophilus* and *M. luteus* in M17 broth [57] and *E. coli, List. monocytogenes, Staph. aureus, Sh. dysenteriae, Ps. aeruginosa* and *Salm. typhi* were propagated in Brain Heart Infusion (BHI) broth. The incubation temperature for the growth of these cultures was 37 °C, except for lactococcal strains (30 °C).

2.3. Screening for bacteriocin-producing strains

All the presumptive enterococcal isolates (1061) were tested for bacteriocin production. Enterococcal isolates were propagated twice in MRS broth. Cell-free culture supernatants (CFCS) of these were prepared according to the method of Cintas et al. [11]. CFCS were obtained from enterococcal strains grown in MRS broth at 37 °C until the early stationary phase ($O.D._{620} = 1.2$). After centrifugation (30 min at 12 000× *g* and 4 °C), the pH of the supernatant was adjusted to 6.0 and the supernatant was heat-treated at 90 °C for 10 min to kill live cells. The antimicrobial activity of the CFCS was determined by spot-on lawn assay [60] with slight modifications. Five microliters of the cell-free supernatants were spotted on TGE agar (1% Tryptone, 2% Glucose, 1% Yeast Extract, 1.5% Agar; [7] plates overlaid with 5 mL

Table I. Bacterial species and strains used in the study.

Bacterial species	Strains and source	Culture conditions
Gram-positive		
Lactococcus lactis subsp. *cremoris*	NCDC 86, Karnal	30 °C, M17
Lactococcus lactis subsp. *lactis*	NCDC 278, Karnal	30 °C, M17
Lactobacillus casei subsp. *casei*	NCDC 017, Karnal	37 °C, MRS*
Lactobacillus brevis	NCDC 001, Karnal	37 °C, MRS*
Lactobacillus acidophilus	NCDC 291, Karnal	37 °C, MRS*
Pediococcus acidilactici	LB-42, USA	37 °C, MRS*
Enterococcus faecalis	DSM 20478, Germany	37 °C, MRS*
Enterococcus faecalis	NCDC 115, Karnal	37 °C, MRS*
Streptococcus thermophilus	NCDC 80, Karnal	37 °C, M17
Staphylococcus aureus	NCDC 110, Karnal	37 °C, BHI**
Listeria monocytogenes	Scott A, USA	37 °C, BHI**
Micrococcus luteus	NCDC 131, Karnal	37 °C, M17
Gram-negative		
Salmonella typhi	NCDC 113, Karnal	37 °C, BHI**
Shigella dysenteriae	NCDC 107, Karnal	37 °C, BHI**
E. coli	NCDC 134, Karnal	37 °C, BHI**
Pseudomonas aerugenosa	NCDC 104, Karnal	37 °C, BHI**

* MRS = de Man, Rogosa, Sharpe.
** BHI = Brain Heart Infusion.

of the TGE soft agar (0.75% agar) seeded with indicator strains (10^5 cfu·mL^{-1})). The plates were kept undisturbed for 2 h and subsequently incubated at 37 °C. After 24 h, the diameters (mm) of the growth inhibition zones were measured. The isolates that did not form any zone were discarded. Enterococcal strains that formed zones against any of the three indicators (*Ped. acidilactici* LB 42, *Ent. faecalis* DSM 20478 and *E. coli* NCDC 134) were considered positive and their antibacterial spectra were determined against a number of both Gram-positive and Gram-negative organisms (Tab. II).

2.4. Sensitivity to enzymes

CFCS were incubated for 2 h at 37 °C with the following enzymes (Sigma Chemical Company, St. Louis, USA.): trypsin, pronase E, α-chymotrypsin, lipase, proteinase K, protease I, protease VIII, papain and catalase to a final enzyme concentration of 1 mg·mL^{-1}. Catalase treatment was performed to avoid possible inhibition by hydrogen peroxide. All the enzymes were dissolved in sterile 50 mmol·L^{-1} phosphate buffer (pH 7.0) at a concentration of 10 mg·mL^{-1}. Controls consisted of samples of enzymes in sterile medium and untreated crude extract. Residual enterocin activity was detected by the spot-on lawn assay as described above, against the indicator strain of *Ped. acidilactici* LB 42.

2.5. Antibiotic susceptibility test

Susceptibility of 60 potent bacteriocin-producing *Enterococcus* strains to the different antibiotics was tested using a Disc Diffusion Assay on Mueller Hinton Agar No. 2 according to the National Committee for Clinical Laboratory Standards [46], using the following antimicrobials (Himedia Laboratories, Pvt. Ltd., Mumbai, India):

Table II. Inhibitory spectra of activity of sixty bacteriocinogenic enterococcal isolates.

S. No	Isolates of Enterococci	Source	*L. monocytogenes*	*M. luteus*	*L. cremoris*	*Strep. thermophilus*	*Lact. casei* subsp. *casei*	*Lact. brevis*	*Lact. acidophilus*	*Ent. faecalis*	*L. lactis* subsp. *lactis*	*Staph. aureus*
1.	**KH 1**	Cream	++	++	–	–	–	++	+++	+++	–	+
2.	**RH 3**	Milk	++	++	–	–	–	++	++	++	–	++
3.	**KH 12**	Cream	+	++	–	–	–	++	++	++	–	++
4.	**KH 24**	Cream	+++	++++	+++	+++	–	+++	+++	+++	+++	–
5.	**DH 28**	*Dahi*	+++	++	–	–	++	++	++	++	–	++
6.	**RH 29**	Milk	+++	++	++	+++	–	++	++	+++	++	+
7.	**RH 31**	Milk	+++	+++	++	+++	–	++	++	+++	++	++
8.	**RH 32**	Milk	+++	+++	++	+++	–	+++	+++	+++	++	+
9.	**RH 33**	Milk	+++	++++	++	+++	–	+++	+++	+++	++	–
10.	**RH 34**	Milk	+++	++	++	++	–	++	++	++	+	++
11.	**RH 38**	Milk	+++	+++	++	+++	–	++	+++	+++	++	+
12.	**RH 39**	Milk	+++	++	++	++	–	++	++	+++	++	+
13.	**RH 40**	Milk	+++	+++	++	+++	–	+++	+++	+++	++	–
14.	**KH 44**	Cream	+++	+++	+++	+++	–	++	+++	+++	++	++
15.	**DH 56**	*Dahi*	++	++	–	–	+	++	++	++	–	++
16.	**DH 57**	*Dahi*	++	++	–	–	+	++	++	++	–	+
17.	**KH 58**	Cream	+++	++	++	+++	–	++	+++	+++	++	+
18.	**DH 59**	*Dahi*	+++	+++	++	++	–	++	++	++	++	+
19.	**CH 60**	Cheddar cheese	+++	+++	++	++	–	++	++	++	++	+
20.	**CH 61**	Cheddar cheese	++	++	++	++	–	++	++	++	++	+
21.	**KH 62**	Cream	+	+	–	–	–	+	+	++	–	–
22.	**KH 64**	Cream	++	–	–	–	–	+	++	+	–	–
23.	**KH 67**	Cream	+	–	–	–	–	+	+	+	–	–
24.	**KH 68**	Cream	–	–	–	–	–	++	++	+	–	–
25.	**KH 70**	Cream	+	++	+	++	–	++	++	+	–	–
26.	**KH 72**	Cream	–	–	–	–	–	+	+	+	–	–
27.	**RH 78**	Milk	++	+	+	++	–	++	++	++	+++	–
28.	**KH 79**	Cream	+++	+++	+++	+++	–	+++	+++	+++	+++	–
29.	**KH 81**	Cream	+++	+++	++	++	–	++	+++	+++	++	–
30.	**RH 83**	Milk	++	+	++	++	–	++	++	++	+	–
31.	**DH 88**	*Dahi*	++	++	–	–	+	+	+	+	++	++
32.	**DH 89**	*Dahi*	++	++	–	–	+	+	+	+	–	++
33.	**FH 90**	Human feces	+++	++	+++	+++	–	++	+++	+++	+++	–
34.	**KH 91**	Cream	–	–	–	–	–	+	+	+	–	+
35.	**DH 92**	*Dahi*	+++	–	–	–	+	+	+	+	–	++
36.	**KH 93**	Cream	++	++	–	–	–	+	+	+	–	+
37.	**FH 96**	Human feces	+++	++	+++	+++	–	+++	+++	+++	+++	–
38.	**KH 97**	Cream	–	–	–	–	–	+	+	+	–	+
39.	**KH 98**	Cream	–	++	–	–	–	+	+	+	–	+
40.	**FH 99**	Human feces	+++	+++	+++	+++	–	+++	+++	+++	+++	–
41.	**FH 100**	Human feces	+++	+++	++	++	–	+++	+++	+++	+	–
42.	**DH 101**	*Dahi*	++	++	+	–	+	++	++	++	–	++
43.	**FH 102**	Human feces	+++	+++	+	++	–	+++	+++	+++	–	–
44.	**RH 106**	Milk	++	++	–	–	+	+	++	+	–	++
45.	**RH 107**	Milk	++	++	–	–	+	+	+	+	–	++
46.	**KH 110**	Cream	++	+	–	–	+	++	++	++	–	++
47.	**KH 111**	Cream	+	–	–	–	–	+	+	+	–	+
48.	**DH 112**	*Dahi*	++	–	–	–	+	+	+	+	–	+
49.	**DH 115**	*Dahi*	+++	++	+++	+	–	+++	+++	+++	++	+
50.	**RH 116**	Milk	++	+++	–	–	+	++	++	++	–	++
51.	**KH 117**	Cream	++	–	–	–	–	+	+	+	–	+
52.	**KH 118**	Cream	++	+	–	–	++	+	+	+	–	+
53.	**KH 126**	Cream	+++	+++	–	–	–	++	++	++	–	–
54.	**FH 129**	Human feces	+++	+++	++	+++	–	+++	+++	+++	+	–
55.	**FH 130**	Human feces	+++	++	+	+	–	++	+++	+++	–	–
56.	**DH 131**	*Dahi*	+	++	–	–	+	++	++	++	–	++
57.	**FH 132**	Human feces	+	+++	+	+	–	+	++	+	+++	–
58.	**FH 133**	Human feces	++	+++	++	+++	–	+++	+++	+++	+++	–
59.	**KH 134**	Cream	+	–	+	–	–	+	+	+	–	+
60.	**CH 135**	Cheddar cheese	+	–	–	–	++	++	+++	+++	–	+

Diameter of inhibitory zone (mm): > 15mm +++; 15–12 ++; <12 +; no zone –.

amikacin (30 μg), ampicillin (10 μg), cefazolin (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), clindamycin (2 μg), co-trimoxazole (25 μg), erythromycin (15 μg), gentamycin (10 μg), ofloxacin (1 μg), penicillin-G (10 units), tetracycline (30 μg) and vancomycin (30 μg). Inhibition zone diameters were measured using slide calipers and results were expressed in terms of resistance (R), moderate susceptibility (MS) or susceptibility (S), according to the interpretative chart recommended by Charteris et al. [10].

2.6. Detection of virulence determinants

2.6.1. Hemolysis

The *Enterococcus* strains were grown overnight in MRS medium at 37 °C, and then transferred onto Blood Agar Base (HiMedia Laboratories, Pvt. Ltd., Mumbai, India) plates containing 5% of fresh calf blood. The plates were incubated for 3 d at 37 °C. The hemolytic reaction was recorded as β-hemolysis if a clear zone of hydrolysis of red blood cells was formed around the colonies, as α-hemolysis, if a green zone appeared around the colonies or as γ-hemolysis if no clear or no green zone of hemolysis appeared around colonies.

2.6.2. DNAse test

All the bacteriocinogenic enterococcal strains were streaked on DNAse agar medium (HiMedia Laboratories, Pvt. Ltd., Mumbai, India) to check their production of DNAse enzyme. Plates were incubated at 37 °C for 48 h. A clear, pinkish zone around the colonies after incubation was considered positive for DNAse production.

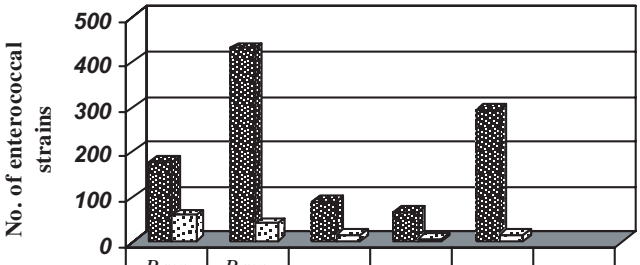
2.6.3. Liquefaction of gelatin

Gelatinase production by selected bacteriocinogenic enterococcal isolates was studied by using TND agar (1.7% tryptone, 0.3% neopeptone, 0.25% dextrose, 0.5% NaCl, 0.25% K_2HPO_4 and 1.5% agar) containing 0.4% gelatin. Petri dishes containing the medium were spot-inoculated with the test culture and incubated at 37 °C for 3 d. The Petri dishes were then flooded with saturated ammonium sulfate solution. Development of clear zones around the inoculated spot against the opaque background indicates a positive reaction.

3. RESULTS

Enterococci were detected in all the samples of raw milk, raw cream, *dahi* and Cheddar cheese, and also in human feces. Enterococcal counts varied from 10^2–10^3 cfu·mL^{-1} (or g^{-1}) in raw milk, Cheddar cheese and *dahi* and 10^4–10^6 cfu·mL^{-1} in raw cream. In human feces, these counts varied from 10^5 to 10^7 cfu·g^{-1}. One thousand and sixty-one enterococcal isolates were randomly selected and screened for the bacteriocin-production characteristic. Out of these, 135 (12.72%) isolates formed zones against at least one of the indicator strains tested. However, among the bac$^+$ isolates, 92.59% of the isolates showed antibacterial (or antimicrobial) activity against *Ped. acidilactici* LB 42, whereas only 45.18% of the isolates were effective against *Ent. faecalis* DSM 20478; but none of the 135 isolates tested was able to inhibit *E. coli* 134. The incidence of the bacteriocin-production characteristic was highest among the cream samples (33.51%), followed by *dahi* samples (15.55%), raw milk samples (9.51%), Cheddar cheese samples (9.2%) and human feces (4.72%) (Fig. 1).

Out of 135 bacteriocin-producing enterococcal strains, 60 (44%) were found to



	Raw cream	Raw milk	Dahi	Cheese	Feces	
Enterococcal strains	179	431	90	65	296	
Bacteriocinogenic enterococcal strains	60	41	14	6	14	

Figure 1. Frequency of incidence of bacteriocin production trait in enterococci from different dairy products and human feces.

be quite potent and stable producers of bacteriocin. These 60 isolates were further characterized for species on the basis of various biochemical tests. Sixteen isolates (26.66%) were identified as *Ent. faecalis*, as observed from their sugar fermentation profiles and their appearance as black-colored colonies on K-tellurite agar. However, the majority of the strains, i.e. 29 (48.33%), were identified as *Ent. faecium*, whereas 5 (8.33%) were identified as *Ent. avium* and the rest (16.66%) belonged to *Ent. raffinosus, Ent. mundtii* or *Ent. saccharolyticus* (Tab. III).

Antibacterial spectra of these sixty bacteriocin-producing enterococcal strains were tested against a large number of Gram-positive organisms *viz. List. monocytogenes, M. luteus, Ent. faecalis, Strep. thermophilus, Lact. brevis*, *Lact. acidophilus, L. lactis* subsp. *cremoris, L. lactis* subsp. *lactis* and *Staph. aureus* and Gram-negative organisms *viz. Salm. typhi*, *Ps. Aeruginosa* and *Sh. dysenteriae.* All the 60 strains of enterococci inhibited *Lact. brevis, Lact. acidophilus* and *Ent. faecalis* strongly. Inhibition of *Listeria* spp. was also the most prevalent type of antagonism (91.66% of bac$^+$ strains), followed by inhibition of *M. luteus* (81.66%), *Staph. aureus* (61.66%), *L. lactis* subsp. *cremoris* (51.66%), *Strep. thermophilus* (48.33%) and *L. lactis* subsp. *lactis* (45%). Among all the Gram-positive bacteria tested, *Lact. casei* subsp. *casei* was found to be least sensitive to the antagonistic activity of the selected strains as 75% of the bac$^+$ strains could not inhibit this strain (Tab. II). Gram-negative organisms such as *Salm. typhi, Sh. dysenteriae* and *Ps. aeruginosa* were not at all inhibited by any of the bacteriocin producers (results not shown).

CFCS were found to be sensitive to trypsin, pronase E, α-chymotrypsin, proteinase K, protease I, protease VIII and papain, but were insensitive to lipase and catalase, showing the protein nature of the inhibitory principle.

None of the 60 bacteriocin-producing enterococcal strains were found to be positive for gelatin liquefaction and DNAse production. However, 9 bacteriocinogenic enterococcal isolates of different origins *viz.* KH 12, RH 34, CH 61, KH 70, FH 102, KH 126, FH 129, FH 130 and DH 131 showed α-hemolysis (Tab. IV). All these strains have a broad spectrum of activity. A few other strains *viz.* RH 40, KH 44,

Table III. Sugar fermentation profiles of bacteriocinogenic enterococcal isolates.

S No.	Isolates of enterococci	Arabinose	Dextrose	Galactose	Inulin	Lactose	Mannitol	Raffinose	Sorbitol	Trehalose	Xylose	Tentative identification
1.	**KH 1**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
2.	**RH 3**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
3.	**KH 12**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
4.	**KH 24**	–	+	+	–	+	+	+	–	+	–	*E. faecium,*
5.	**DH 28**	–	+	+	–	+	+	–	+	+	–	*E. raffinosus, E. mundtii, E. saccharolyticus*
6.	**RH 29**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
7.	**RH 31**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
8.	**RH 32**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
9.	**RH 33**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
10.	**RH 34**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
11.	**RH 38**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
12.	**RH 39**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
13.	**RH 40**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
14.	**KH 44**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
15.	**DH 56**	–	+	+	–	[+]	+	–	+	+	–	*E. avium**
16.	**DH 57**	–	+	+	–	+	+	–	+	+	–	*E. avium**
17.	**KH 58**	–	+	+	–	[+]	+	+	–	+	–	*E. faecium, E. casseliflavus*
18.	**DH 59**	–	+	+	–	+	+	+	–	+	–	*E. faecium, E. casseliflavus*
19.	**CH 60**	–	+	+	–	[+]	+	+	–	+	–	*E. faecium, E. casseliflavus*
20.	**CH 61**	–	+	+	–	+	+	+	–	+	–	*E. faecium, E. casseliflavus*
21.	**KH 62**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
22.	**KH 64**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
23.	**KH 67**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
24.	**KH 68**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
25.	**KH 70**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
26.	**KH 72**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
27.	**RH 78**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
28.	**KH 79**	–	+	+	–	[+]	+	+	–	+	–	*E. faecium, E. casseliflavus*
29.	**KH 81**	–	+	+	–	+	+	+	–	+	–	*E. faecium, E. casseliflavus*
30.	**RH 83**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
31.	**DH 88**	–	+	+	–	+	+	+	+	+	–	*E. raffinosus, E. mundtii, E. saccharolyticus*
32.	**DH 89**	–	+	+	–	+	+	+	+	+	–	*E. raffinosus, E. mundtii, E. saccharolyticus*
33.	**FH 90**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
34.	**KH 91**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
35.	**DH 92**	–	+	+	–	+	+	–	+	+	–	*E. avium**
36.	**KH 93**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
37.	**FH 96**	–	+	+	–	+	+	+	–	+	–	*E. faecium, E. casseliflavus*
38.	**KH 97**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
39.	**KH 98**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
40.	**FH 99**	–	+	+	–	+	+	+	–	+	–	*E. faecium, E. casseliflavus*
41.	**FH 100**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
42.	**DH 101**	–	+	+	–	+	+	+	+	+	–	*E. raffinosus, E. mundii, E. saccharolyticus*
43.	**FH 102**	–	+	+	–	+	+	+	–	+	–	*E. faecium, E. casseliflavus*
44.	**RH 106**	–	+	+	–	+	+	+	+	+	–	*E. raffinosus, E. mundtii, E. saccharolyticus*
45.	**RH 107**	–	+	+	–	+	+	+	+	+	–	*E. raffinosus, E. mundtii, E. saccharolyticus*
46.	**KH 110**	–	+	+	–	+	+	+	+	+	–	*E. raffinosus, E. mundtii, E. saccharolyticus*

Table III. Continued.

S No.	Isolates of enterococci	Arabinose	Dextrose	Galactose	Inulin	Lactose	Mannitol	Raffinose	Sorbitol	Trehalose	Xylose	Tentative identification
47.	**KH 111**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
48.	**DH 112**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
49.	**DH 115**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
50.	**RH 116**	–	+	+	–	+	+	–	+	+	–	*E. avium**
51.	**KH 117**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
52.	**KH 118**	–	+	+	–	+	+	–	+	+	–	*E. avium**
53.	**KH 126**	–	+	+	–	+	+	–	+	+	–	*E. faecalis*
54.	**FH 129**	–	+	+	–	+	+	–	–	+	–	*E. faecium*
55.	**FH 130**	–	+	+	–	[+]	+	–	–	+	–	*E. faecium*
56.	**DH 131**	–	+	+	–	+	+	+	+	+	–	*E. raffinosus*, *E. mundtii*, *E. saccharolyticus*
57.	**FH 132**	–	+	+	–	[+]	+	–	–	+	–	*E. faecium*
58.	**FH 133**	–	+	+	–	+	+	+	–	+	–	*E. faecium*, *E. casseliflavus*
59.	**KH 134**	–	+	+	–	+	+	+	–	+	–	*E. faecalis*
60.	**CH 135**	–	+	+	–	+	+	+	+	+	–	*E. raffinosus*, *E. mundtii*, *E. saccharolyticus*

* Sugar fermentation profile similar to *E. faecalis* but tentatively designated as *E. avium* because these are K-tellurite –ve.

KH 62, KH 70, RH 78 and FH 90 showed weak α-hemolysis, while no hemolytic activity was observed for the rest of the strains tested. Furthermore, incidence of α-hemolysis was not found to be source-dependent.

Antibiograms of sixty bacteriocinogenic enterococcal strains were determined by using a Disc Diffusion Assay. All the sixty strains of enterococci possessing the bacteriocin-production characteristic were sensitive to ofloxacin and 93.33% of the strains were sensitive to vancomycin. Only 6.67% of the isolates were resistant to chloramphenicol and 10% of the isolates were resistant to tetracycline, co-trimoxazole and ampicillin. Resistance of bacteriocinogenic enterococci was observed for cephalothin and cefuroxime (88.33%), followed by their resistance to amikacin (70%), erythromycin (46.67%), clindamycin (33.33%), penicillin G (25%) and gentamicin (23.33%). Out of a total of four vancomycin-resistant strains, two strains were from raw milk and the other two were from cream samples. Nine moderately resistant enterococcal isolates were from cream samples and one was from a *dahi* sample. However, none of the isolates from human feces was found to be resistant to vancomycin (Fig. 2).

4. DISCUSSION

Enterococci form part of LAB, that are of great importance in foods. These organisms have been implicated in spoilage of processed meats; however, they are important in ripening and aroma development of certain traditional cheeses and sausages and are intentionally used as starter cultures or as culture adjuncts for the manufacture of certain types of cheeses [21, 23, 28, 48, 55]. Levels of enterococci in different cheese curds have been reported to range from 10^4 to 10^6 cfu·g^{-1} and in fully ripened cheeses from 10^5 to 10^7 cfu·g^{-1} [25, 27, 39]. In humans, enterococci, along with approximately 450 other aerobic and anaerobic bacterial species, form a part of the normal intestinal flora. In most individuals, 10^5–10^7 cfu of enterococci per gram in stools have been reported [44].

Enterococci have drawn research interest because of their presence almost everywhere in the food chain, as well as in the

Table IV. Study of incidence of virulence traits in sixty bacteriocinogenic enterococci isolates.

S. No.	Isolate No.	Gelatin production	Hemolysin production	DNase production
1.	**KH 1**	–	γ	–
2.	**RH 3**	–	γ	–
3.	**KH 12**	–	α	–
4.	**KH 24**	–	γ	–
5.	**DH 28**	–	γ	–
6.	**RH 29**	–	γ	–
7.	**RH 31**	–	γ	–
8.	**RH 32**	–	γ	–
9.	**RH 33**	–	γ	–
10.	**RH 34**	–	α	–
11.	**RH 38**	–	γ	–
12.	**RH 39**	–	γ	–
13.	**RH 40**	–	[α]	–
14.	**KH 44**	–	[α]	–
15.	**DH 56**	–	γ	–
16.	**DH 57**	–	γ	–
17.	**KH 58**	–	γ	–
18.	**DH 59**	–	γ	–
19.	**CH 60**	–	γ	–
20.	**CH 61**	–	α	–
21.	**KH 62**	–	[α]	–
22.	**KH 64**	–	γ	–
23.	**KH 67**	–	γ	–
24.	**KH 68**	–	γ	–
25.	**KH 70**	–	[α]	–
26.	**KH 72**	–	α	–
27.	**RH 78**	–	[α]	–
28.	**KH 79**	–	γ	–
29.	**KH 81**	–	γ	–
30.	**RH 83**	–	γ	–
31.	**DH 88**	–	γ	–
32.	**DH 89**	–	γ	–
33.	**FH 90**	–	[α]	–
34.	**KH 91**	–	γ	–
35.	**DH 92**	–	γ	–
36.	**KH 93**	–	γ	–
37.	**FH 96**	–	γ	–
38.	**KH 97**	–	γ	–
39.	**KH 98**	–	γ	–
40.	**FH 99**	–	γ	–
41.	**FH 100**	–	γ	–
42.	**DH 101**	–	γ	–
43.	**FH 102**	–	α	–
44.	**RH 106**	–	γ	–
45.	**RH 107**	–	γ	–
46.	**KH 110**	–	γ	–
47.	**KH 111**	–	γ	–
48.	**DH 112**	–	γ	–
49.	**DH 115**	–	γ	–
50.	**RH 116**	–	γ	–
51.	**KH 117**	–	γ	–
52.	**KH 118**	–	γ	–
53.	**KH 126**	–	γ	–
54.	**FH 129**	–	α	–
55.	**FH 130**	–	α	–
56.	**DH 131**	–	α	–
57.	**FH 132**	–	α	–
58.	**FH 133**	–	γ	–
59.	**KH 134**	–	γ	–
60.	**CH 135**	–	γ	–

[α] = Very weakly α-hemolytic.

environment, and also because of their use in the production of probiotics and other fermented foods. Besides this, bacteriocin production is also a common characteristic among enterococci and is responsible for the inhibition of food spoilage and pathogenic organisms [4,14,25,30,35]. Enterococci are, therefore, interesting as protective cultures for cheese manufacture and also for the preparation of novel probiotics [17, 21, 49]. However, severe safety criteria must be established to guarantee a safe, commercial use of enterococci. Considering their clinical involvement in infections and antibiotic resistance [2, 42], the safety of *Ent. faecium* and *Ent. faecalis* strains associated with food fermentation and probiotics is being questioned. Therefore, it becomes imperative to evaluate the bacteriocin-producing enterococcal strains for their virulence determinants and vancomycin resistance.

Bacteriocin production was observed mainly among the strains of *Ent. faecalis* and *Ent. faecium*, along with some other species of enterococci. These species encompassed strains of different origins covering dairy food isolates (60 strains from raw milk, 41 from cream, 14 from *dahi* and 6 from Cheddar cheese) and human feces (14 isolates). These findings are further corroborated by other studies showing that bacteriocin-producing enterococci can exist in diverse environments and are also found associated with the gastrointestinal tract of humans [15, 27, 41]. Among *Enterococcus* strains of human origin, *Ent. faecium* (16.67%) displayed the highest bacteriocinogenic activity. Reports showing isolation of bacteriocinogenic enterococci from a number of different sources e.g. meat products, black olives, infant stools, soy beans, goat's cheese, yellow cheese from sheep's milk, fermented products from mare's milk and corn silage [1,3, 5, 20, 22, 35, 37, 49, 54] confirm their ubiquitous occurrence and persistence. Enterococcal strains were found to exhibit a wide

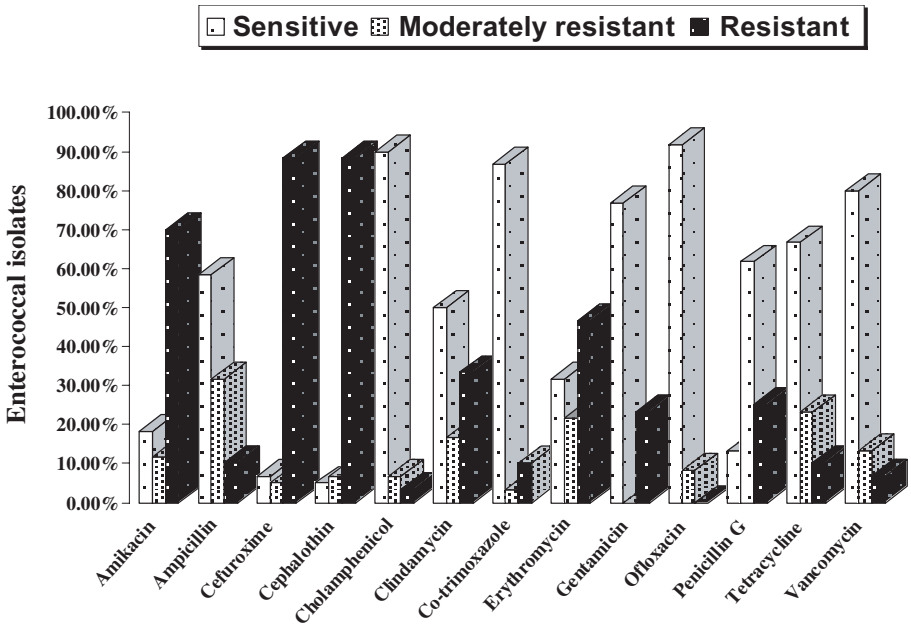


Figure 2. Antibiogram of bacteriocinogenic enterococcal isolates.

range of activity against Gram-positive organisms but no correlation could be established between the source of isolation of the strains and the inhibitory spectrum. Importantly, enterocins show a strong activity against *Listeria*, which can be of practical use in the food industry [18, 26, 35]. *Enterococcus* strains displaying an inhibitory spectrum due to the production of enterocins targeted against *Listeria* and/or *Clostridium* [30, 59] have been reported to be interesting as protective cultures for cheese manufacture [48]. While a few reports have shown enterocins to be active against Gram-negative bacteria such as *E. coli* [5, 26, 49, 58], *Shigella sonnei, Shigella flexeneri* [54] and *Vibrio cholera* [52], the majority of the reports show that bacteriocins are least active towards Gram-negative organisms [3, 6, 12, 37]. In the present study none of the enterocins was found to be effective against Gram-negative organisms.

It is imperative that the *Enterococcus* strains, in particular those showing a broad spectrum of activity and having potential application in foods, be checked for the presence of virulence characteristics. Accordingly, sixty bacteriocinogenic *Enterococcus* strains exhibiting broad spectrum activity were evaluated carefully for the presence of hemolysins, gelatinase and DNAse production. Cytolysin/hemolysin activity is seen as a potent virulence factor in several animal model studies [32, 34]. No β-hemolysis was observed in *Ent. faecium* or in *Ent. faecalis* strains of dairy or fecal origin, thus apparently showing the absence of the cytolysin gene in the bacteriocin-producing *Ent. faecalis* and *Ent. faecium* strains. Alpha-hemolysis among enterococcal strains may usually be confronted, but it does not pose a serious threat as other virulence characteristics do. Absence of hemolytic activity should be a selection criterion for

(bacteriocin-producing) starter strains of dairy use [28, 30]. Nevertheless, absence of hemolytic activity in enterococci isolated from food does not mean that these bacteria are non-virulent [23, 24]; a thorough understanding of all the characteristics will certainly contribute to the safety evaluation of enterococci. Besides testing for hemolysin production, the isolated bacteriocinogenic strains of enterococci were tested for gelatinase production and DNAse production, that may have a role in virulence [53,56]. However, none of the strains either of food or human origin was found to possess any of these virulence traits. Such strains may not, therefore, pose any problem for their use in foods, as also reported by other authors [9, 21, 25, 38].

The incidence of antibiotic-resistant enterococci (ARE) is quite widespread in foods. They have been isolated from meat products, dairy products and ready-to-eat foods, and even within enterococcal strains used as probiotics [24, 27]. The role of ARE, especially VRE (vancomycin-resistant enterococci), has been emphasized by Giraffa [27], as possible natural food reservoirs in the dissemination of antibiotic resistance in the environment. For safety reasons, sensitivity of commercially exploitable strains against commonly used antibiotics is desirable in order to be used as co-cultures or starter cultures. Examples of acquired resistance through exchange of resistance-encoding genes are resistance to tetracycline, chloramphenicol, glycopeptides and vancomycin. In this study, the majority of strains were found to be sensitive to these antibiotics. Giraffa and Sisto [29] did not find VRE among the strains isolated from Italian cheeses. In contrast, high frequencies of ARE and VRE have been reported among isolates from meat [38].

In conclusion, enterococci are ubiquitous in nature and are present in dairy products and the gut of humans. Their capacity to produce bacteriocins active against spoilage or pathogenic bacteria, particularly against *Listeria*, might be a powerful tool for the protection of certain dairy products and meat products against such pathogens. However, some enterococci carry virulence factors, but incidence of these factors in the enterococcal isolates of food or human origin is almost negligible. Antibiotic-resistant strains of enterococci that are also bacteriocin producers may be encountered in some dairy foods and the human intestine but their frequency of incidence is much less. Nevertheless, a careful evaluation of the commercially exploitable strains may be very helpful for the food industry for their safe use.

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