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ENTEROCOCCAL ISOLATES FROM RAW MILK AND DAIRY PRODUCTS IN RIYADH REGION AND THEIR SUSCEPTIBILITY TO COMMON ANTIBIOTICS

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تم في هذا البحث جمع ١٠٠ عينة من الحليب الخام وكذلك أنواع من الجبنة واللبن الزبادي من المحلات الخاصة والأسواق في منطقة الرياض. كما تم إجراء التحليل البكتيريولوجي للتعرف على أنواع المكورات السبحية المعوية (Entirococci) وأنواع بكتريا حمض اللاكتيك وذلك عن طريق عمل تخفيفات مختلفة من العينات وزراعتها هوائيا على بيئة أجار الدم من (الخراف) وبيئة Edwards مضاف اليها الدم والتحضين لمدة يوم عند درجة حرارة ٣٧ ْم. هذا وقد تم تعريف ١٢٥ عزله من البكتيريا التي تم تصنيفها إلي مجموعات حسب الشكل الظاهري والخواص المورفولوجية وصبغة جرام واختبار الكتاليز كما تم تصنيفها سيرلوجيا حسب طريقة Lancefield grouping.

وقد اظهرت النتائج أن هناك ٨٨ عزله من نوع الانتيروكوكس فيشيم أي بنسبة ٧٠% من الأنواع المعزولة و٢٦ عزله من الانتيروكوكس فيكالس بنسبة ٢١% ، بالإضافة إلى ١١عزله من الأنواع الأخرى من بكتيريا حمض اللاكتيك أي بنسبة ٩%من العينات المعزولة والتي تمثل ٣ عزلات من الانتيروكوكس جالينارم و٣ عزلات من الانتيروكوكس ديورانز و٥ عزلات من الايروكوكس فيريدانز.

كما اظهرت النتائج أن هناك تأثيرا واضحا للحموضة والملوحة على مدى عزل وعدد البكتيريا المعزولة في العينات المختبرة فكلما قلت الحموضة في عدم وجود الملح أو قلته ظهر النوع أنتيروكوكس فيكالس وبكتيريا حمض اللاكتيك بكثرة والعكس بالعكس.

وعلي العكس من ذلك لم يكن لدرجة الحموضة والملوحة تأثير بالغ على نوع انتيروكوكس فيشيم والتي ظهرت عند جميع درجات الحموضة في جميع العينات المختبره مما يعكس مدى انتشارها في البيئة ومقاومتها للظروف البيئية الصعبة من حيث الحرارة والحموضة أو تركيز الملح وبالتالي سهولة نقلها من شخص إلي آخر. كذلك فان وجود وانتشار هذا النوع من الانتيروكوكاي غالبا ما يكون بسبب قدرتها على النمو على مدى واسع من درجات الحرارة ومقاومتها للحرارة وزيادة الحموضة أو تركيز الملح.

كذلك تم تقدير التركيز المثبط (MIC) لعدد ٩من المضادات الحيوية الشائعة لكل عزله من إجمالي الـــ ١٢٠ عزلة المختبرة. والتي تم تقديرها بواسطة تخفيفات مختلفة لكل مضاد حيوي علي بيئة اجار الدم Muller Hinton.

وقد أظهرت النتائج أن جميع العزلات حساسة للمضاد الحيوي الامبسليين (AM) ما عدا سلالة واحدة من الانتيروكوكس فيكالس. بينما اظهر مضاد اريثروميسين (EM) فاعلية جيده حيث كان التركيز المثبط لـــ ٥٠% و ٩٠% من العزلات ١/١ لميكروب جالينارم و ٤/١ لميكروب الانتيروكوكس فيكالس و ٢/٨ لميكروب الانتيروكوكس فيشيم و ٤/٤ لميكروب الانتيروكوكس ديورانز أو ايروكوكس فيريدانز ميكروجرام/مل.

كما أظهرت جميع العزلات مقاومة للمضاد الحيوي سيفوكستين (CF) وأن حوالي ٥٠% منها كان أيضا مقاوم لكل من كلورامفينيكول (CM)، تتراسيكلين (TC)، او مركب تراي ميثوبريم/سلفاميثوكسازول (SXT) فمثلا عند نقطة الفصل لحساسية التتراسيكلين (< ١٦ ميكروجرام / مليليتر) كان معدل المقاومة لهذا المضاد ١٦% لميكروب الانتيروكوكس فيكالس و ٣٥% لميكروب انتيروكوكس فيشيم و ١٠٠% لميكروب الانتيروكوكس جالينارم. بينما كان معدل مقاومة هذه الميكروبات لمضاد الفانكوميسين (VM) هو ٤٤% و١٩% و ١٠٠% على التوالي.

وهذه النتائج قد تقودنا للإستنتاج أنه ربما يكون هناك علاقة وثيقة بين هذه المصادر ومدى مقاومة هذه الميكروبات للمضادات الحيوية في الإنسان.

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A total of 100 samples of raw milk, various cheeses, labnah, yogurt, and egett were collected from appropriate suppliers and markets in Riyadh region. Bacteriological analysis for typing of enterococci and other lactic Acid Bacteria (LAB) was carried out by plating appropriate dilutions of each sample on sheep blood agar and Edwards blood agar plates. After overnight aerobic incubation at 37°, the presumptive identification was done by colony morphology, cultural characteristics, Gram-stain and catalase production. Final identification to the genera and species level of the total 125 bacterial isolates was completed by API-20 strips as well as Lancefield-serogrouping. Results revealed that Enterococcus faecium (88 isolates) accounts of 70% of total bacterial isolates, while Enterococcus faecalis (26 isolates) accounts of up to 21% and other LAB constituted about 9% of total recovered isolates. The later isolates comprises 3, 3, and 5 isolates of Enterococcus gallinarum, Enterococcus durans, and Aerococcus viridans respectively.

The results revealed that nature of sample, its pH, and salinity clearly affect the incidence and number of recovered bacterial isolates. Thus as pH rises towards neutrality, with no salt or low salinity, E. faecalis and other LAB were recovered more frequently, and vice versa. In contrast, E. faecium was routinely isolated from most of the examined samples regardless of their pH range and salinity- content, reflecting its ubiquitous nature and its tolerance to drastic environmental conditions, thereby facilitating person to person transmission. The dominance or persistence of enterococci in examined samples is most probably attributed to their wide range of growth temperatures, their tolerance to heat, salt and acid.

In addition, the MIC of each of the tested 120 isolates was determined by serial dilution in Muller Hinton sheep blood agar against 9 antibiotics. All isolates were sensitive to ampicilin with the exception of one E. faecalis strain that showed an MIC of 4 ug/ml. While Erythromycin (EM) exhibited also a good activity with an MIC50/MIC90 of 1/1, 1/4, 2/8 and 4/4 ug/ml. for E. gallinarum, E. faecalis, E. faecium, and E. durans or Aerococcus viridans isolates respectively. Whereas all isolates were resistant to cefoxitin and about 50% were also resistant to Chloramphenicol (CM), Tetracycline (TC), or Trimethoprim / Sulfamethoxazole (SXT). Thus at the breakpoint of MIC (>16 ug/ml.) (TC) resistance rate for E. faecalis was 16% and for E. faecium and E. gallinarum was 35% and 100% respectively. Whereas that for vancomycin (VM) the figures were 44%, 19% and 100% respectively. It is concluded that the examined samples may constitute a potential source for the dissemination of antibiotic resistant determinants to human.

INTRODUCTION

Many streptococci form part of the normal flora of humans and animals, and live harmlessly as commonsals while others may cause diseases in humans and animals. In traditional taxonomic schemes, the *Streptococci belong* to the family streptococcaceae.[1] Lancefield[2] detected a series of group of antigens that also made possible the sub classification of some streptococci. The antigens detected[3] in the Lancefield grouping system are either cell wall polysaccharides (as in human group A, B, C, F, and B streptococcus species). On the other hand, enterococci are used as probiotics to improve the microbial balance of the intestine in humans and animals.[3,4] In certain cheese, they are significant in repening and the development of flavor.[5] Furthermore enterococci have emerged in recent years as pathogens in growing number of serious nosocomial infections including bacteremia and interabdominal and urinary tract infections.[6] Accordingly, the present study was under taken to determine the species and incidence of enterococcal isolates and other lactic acid isolates from raw milk and dairy products and their susceptibility to common antibiotics.

MATERIAL AND METHODS

Sampling

A total of 100 different cheeses and raw milk samples were collected from various markets and farms in Riyadh city, Saudi Arabia. The samples were transferred to the laboratory and kept in refrigerator at 4° till the bacterological analysis. The kind and number of raw milk or cheese samples as well as country of origin are shown in Table (1).

Bacterial isolates

Samples of milk or cheese inquestion (10 ml or 10 gm) were aseptically homogenized in Todd-Hewitt (TW) broth (90 ml) medium then incubated overnight at 37°. After incubation dilutions were made and subcultured on Blood agar and Edwards Blood agar (Oxiod, UK) media. The inoculated plates were then incubated aerobically at 37° for 24 hours.[7]

Representative colonies were then purified by streaking on the same media and identified by convential cultural characteristics and identify confirmation by API-20 system according to El-Kersh *et al.*[7] and Facklam & Gollins[8] as well as Lancefield serogrouping coagglantination tests (Denka Senka, Tokyo, Co. Ltd., Japan).

Minmal Inhibitory Concentration (MICs) Of recovered bacterial isolates

Minimal inhibitory concentrations (MICs) for nine antibiotics (AM, CH, GM, CL, VM, EM, SXT, TC, CF) were determined using two fold serial dilution of the antibiotics in Mueller-Hinton agar (Oxiod, UK) by the standard methods.[9] The agar plates were inoculated using a multi-point inoculator (Denley, UK). Approximately 10^4 CFU/spot of the appropriately diluted overnight broth cultures were inoculated. Plates were incubated at 37° for 18 hours. The MICs of the antibiotics were defined as the lowest concentration at which no growth was detected. Standard quality control strains (*E. faecalis* ATCC 29212, and *S. aureus* ATCC 29213) were included in each run.[6]

RESULTS AND DISCUSSION

The analysis of lactic acid bacterial isolates (LAB) from raw milk and dairy products revealed that the examined samples were rich with these isolates as expected. Thus a total of 125 LAB isolates were recovered from the 100 samples tested and originated from local and foreign suppliers (Table 1). The isolate-presumptive identification was carried out by colony morphology, cultural characteristics, gram stain and catalase test. Lancsfield grouping and API identity confirmation results distinguished these isolates into 88 *E. faecium*, 26 *E. faecalis*, and 5, 3 and 3 isolates of *Aerococcus viridans*, *E. gallinarum*, and *E. durans* respectively (Table 2). These percentages of genera and species incidence may reflect their intrinsic tolerance towards variation in environmental conditions, and physiological capability of proteolysis activity, acid production from sugars, thereby pH variation, salt concentrations, as well as milk fat hydrolysis by esterase.[10] The obtained results suggest that as pH rises toward neutrality, *E. faecalis* and other LAB were recovered more frequently and vice versa. This holds also true as the cheese salinity decreases. In contrast *E. faecium* was routinely isolated from most of the examined samples regardless of pH range and salt concentration.

The low incidence of *E. faecalis* recovery from raw milk despite its neutral pH, (only one isolate from 24 samples) may suggest the good hygienic measures of raw milk handling in Saudi Arabia.

It should be mentioned, that enterococci exhibit higher proteolysis activity than other LAB and this is considered important for cheese ripening aroma.[11] Similarly *E. durans* was also shown to be important for aroma development in Feta cheese.[12] Obviously enterococci and other LAB play an important role in the manufacture of cheese typical of some regions and their use impact on this part of the dairy-industry. The dominance or persistence of enterococci in tested dairy product samples can be attributed to their wide range of growth temperatures, their high tolerance to heat, salt and acids.[10]

The MICs required to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of the tested 120 isolates and their respective resistance (%) to the tested antibiotics are listed in Table (3). All bacterial isolates were sensitive to the beta lactam AP with the exception of one *E. faecalis* strain that exhibited an elevated MIC of 4 ug/ml. Most isolates showed moderate to high resistance toward CL, but comparatively good susceptibility towards EM with an MIC_{50}/MIC_{90} range of 1/1 ug/ml to 2/8 ug/ml. These findings are in general agreement with those previously reported,[13,14] from USA and UK respectively; but lower than those of a local study[6] in Saudi Arabia where EM exhibited poor activity with a resistance rate of 44% against clinical enterococcal isolates. Table (3) also shows that

Table 1: Kind and number of raw milk, cheese samples and country of origin.

No.	Type of cheese	No. of sample	Producing Country	
1	Raw Milk	24	Saudi Arabia	(24)
2	White Cheese	12	Denmark	(4)
			Bulgaria	(2)
			Hangaria	(2)
			Turkey	(1)
			Egypt	(1)
			France	(1)
			Germany	(1)
3	White Cheese (Low Salt)	12	Egypt	(5)
			Saudi Arabia	(2)
			Denmark	(1)
			Bulgaria	(1)
			Greece	(1)
			Hangaria	(1)
			France	(1)
4	Cream Cheese (Double)	11	Egypt	(7)
			Denmark	(1)
			Hangaria	(1)
			France	(1)
			Hungary	(1)
5	Labnah	8	Saudi Arabia	(3)
			Turkey	(2)
			Egypt	(1)
			France	(1)
			Lebanon	(1)
6	Fresh Cream	6	Saudi Arabia	(5)
			Egypt	(1)
7	White Cheese (Domyati)	4	Egypt	(4)
8	White Cheese (Hallom)	3	Syria	(2)
			Saudi Arabia	(1)
9	White Cheese (Arish)	3	Egypt	(3)
10	White Cheese (Akawi)	2	Syria	(1)
			Egypt	(1)
11	White Cheese (Akawi Free Salt)	2	France	(1)
			Syria	(1)
12	White Cheese (Feta)	2	Denmark	(1)
			France	(1)
13	White Cheese (Bader)	2	Egypt	(2)
14	White Cheese (Low Fat)	2	Holland	(1)
			Denmark	(1)
15	Yogurt	2	Saudi Arabia	(2)
16	White Cheese (Free Salt)	1	Denmark	(1)
17	White Cheese (Free Fat)	1	Denmark	(1)
18	White Cheese (Shillal)	1	Syria	(1)
19	White Cheese (Akawi Low Salt)	1	France	(1)
20	Egett	1	Saudi Arabia	(1)
Total		100		

Table 2: Raw milk and dairy product, pH range, number of bacterial isolates and % of total species.

Kind of cheese	pH rang	Bacterial Isolates	No. of isolates	% of the species
Raw Milk (24 samples)	7	*E. faecium* *E. faecalis* *Aerococcus viridans*	21 1 1	24 3 20
White cheese (12)	5→6	*E. faecium* *E. faecalis* *E. gallinarum*	15 2 2	17 8 67
White cheese (Low salt) (12)	5→7	*E. faecium* *E. faecalis* *E. durans*	10 4 1	11 15 33
White cheese (Double) (11)	5→6.5	*E. faecium* *E. faecalis* *E. gallinarum*	7 3 1	8 11 33
Labnah (8)	4→5.5	*E. faecium* *E. faecalis*	9 2	10 8
Fresh Cream (5)	6→7	*E. faecium* *E. faecalis* *E. durans* *Aerococcus viridans*	2 4 1 1	2 15 33 20
White cheese (Domyati) (4)	4→6.5	*E. faecium* *E. faecalis* *Aerococcus viridans*	5 1 1	6 4 20
White cheese (Hallom) (3)	6→5.7	*E. faecium* *E. faecalis*	4 1	4 4
White cheese (Arish) (3)	4→4.5	*E. faecium* *E. faecalis* *E. durans*	2 1 1	2 4 33
White cheese (Akawi) (2)	7	*E. faecalis* *Aerococcus viridans*	2 1	8 20
White cheese (Akawi Low Salt) (1)	6	*E. faecium*	3	3
White cheese (Akawi Free Salt) (2)	5→6.5	*E. faecium* *E. faecalis*	1 1	1 4
White cheese (Feeta) (2)	5→6	*E. faecium*	2	2
White cheese (Bader) (2)	5	*E. faecium* *E. faecalis*	2 1	2 4
White cheese (Low fat) (2)	6→6.5	*E. faecium* *E. faecalis*	1 1	1 4
Yogurt (2)	4	*E. faecium*	2	2
White cheese (Free salt) (1)	7	*E. faecium*	1	4
White cheese (Free Fat) (1)	5	*E. fecalis*	1	4
White cheese (Shillal) (1)	5	*E. fecalis* *Aerococcus viridans*	1 1	4 20
Egett (1)	5	*E. faecium*	1	1

Table 3: Minimum inhibitory concentrations of antibiotics against recovered bacterial isolates.

Type of bacteria isolates (No.)	MIC_{50} / MIC_{90} Antibiotics * in Mg/Liter (R%)								
	AP	CL	CM	EM	GM	VM	SXT	TC	CF
E. faecium (85)	(0.0)	(45)	(44)	(31)	(26)	(19)	(38)	(35)	(100)
MIC_{50}	1	1	4	2	8	2	2	4	≤ 8
MIC_{90}	1.5	8	8	8	16	8	8	64	≤ 8
E. faecalis (25)	(4)	(72)	(44)	(28)	(16)	(44)	(24)	(16)	(100)
MIC_{50}	1	8	4	1	4	4	1	4	< 8
MIC_{90}	2	8	8	4	16	16	4	64	< 8
E. gallinarum (3)	(0.0)	(100)	(100)	(0.0)	(0.0)	(100)	(100)	(100)	(100)
MIC_{50}	0.5	8	8	1	4	16	8	64	> 8
MIC_{90}	1	8	8	1	4	16	8	64	> 8
E. durans (3)	(0.0)	(0.0)	(66)	(66)	(0.0)	(0.0)	(66)	(0.0)	(100)
MIC_{50}	0.5	1	8	4	8	2	8	≤ 4	> 8
MIC_{90}	1	1	8	4	8	2	8	≤ 4	> 8
Aerococcus Viridans (4)	(0.0)	(100)	(100)	(75)	(0.0)	(0.0)	(0.0)	(0.0)	(100)
MIC_{50}	1	8	8	4	8	4	≤ 1	≤ 4	> 8
MIC_{90}	1	>8	8	4	8	4	≤ 1	≤ 4	>8

∗The breakpoints (ug / ml): Ampicillin (AP), ≤ 0.25 ≥ 4; Erythromycin (EM) and Clindamycin (CL), ≤ 1 ≥ 4; Gentamicin (GM) and Tetracycline (TC), ≤ 8 ≥ 16; Vancomycin (VM) and Chloramphenicol (CM), ≤ 4 ≥ 8; Cefoxitin (CF), ≤ 1 ≥ 8; Trimethoprim / Sulfamethoxazole (SXT), ≤ 2 ≥ 4.

GM exhibited an MIC_{50}/MIC_{90} range of 4/4 ug/ml to 8/16 ug/ml for the tested isolates and none of the isolates exhibited high-level (>2000 ug/ml) of GM resistance. All isolates, however were resistant to CF as judged by the breakpoint of susceptibility (MIC<8 ug/ml).

In agreement with Warren[16] chloramphenicol exhibited a resistance rate of 44% for both *E. faecium* and *E. faecalis*, but with increased resistance rates against other LAB isolates, with 66% resistance for *E. durans* and 100% for *Aerococcus viridans* or *E. gallinarum*. This may be attributed to the extensive use of this drug and its congers in animal husbandries.[5] This holds also true for SXT combination[6] and TC which showed full resistance (100%) against *E. gallarinum* Boyce[17] demonstrated a progressive increase in resistance to TC among enterococci between 1990 and 1992.

It is evident also from Table (3) that in general**,** *E. faecium* was more resistant to most of the tested antibiotics with the exception of VM and AP which showed less activity against *E. faecalis*. The resistance percentages of both drugs were 19 & 0.0% for *E. faecium* and 44 & 4% for *E. faecalis* respectively. Their MIC_{50}/MIC_{90} against *E. faecium* were 1/1.5 and 2/8 ug/ml, whereas those for *E. faecalis* were 1/2 and 4/16 ug/ml respectively. High susceptibilities (90% and 100%) to AP were also demonstrated from different places.[14,18] On the other hand, resistance rates as high as 75% to AP were reported from a study from France between 1985and 1993.[19]

The obtained rate of VM resistance (breakpoint >16 ug/ml) appears to be higher than those of previous local studies on clinical isolates of entrococci of Al-Auaji *et al.*[6] and Qadri *et al.*[20] with resistance rates to VM of 11 and 3% respectively, but comparable resistance rates, were also demonstrated in other studies.[21,22]

It should be emphasized that vancomycin resistant enterococci (VRE) are also highly resistant to all standard anti-enterococcal drugs, including penicillin-aminoglycoside combinat-ions, leaving only a few alternatives for

successful treatment. The VRE are therefore considered as a serious risk group among bacterial nosocomial pathogens.[5,6]

Furthermore, *E. faecalis* accounts of 85-90% of the clinical entrococcal isolates, and *E. faecium* and other species represent 5-10% and not more than 5% respectively.[23,24,25-32] This situation is complicated by the fairly common trait of transferable drug resistance within the two enterococcal species, which may confer resistance especially the acquired resistance phenotypes (Van A & B) to glycopeptides (VRE) are transferable by conjugation.[15]

The importance of VRE in nosocomial disease, therefore, cannot be disregarded. Although *E. faecalis* seems to have a greater pathogenic potential than *E. faecium*, the association of either of these species with food may not be considered desirable.[10]

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