# Antibiotic resistance gene in dairy starter culture and probiotic bacteria-A review

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**Abstract:** Lactic acid bacteria (LAB) are intrinsically resistant to many antibiotics. Moreover, some LAB is highly adaptable creatures and are capable of developing resistance to antibiotics. The antibiotic resistance can cause significant danger and suffering for many people with common bacterial infections, those once easily treated with antibiotics. In many cases resistances are transmissible; therefore no particular safety concern is associated with intrinsic type of resistance. Plasmid-associated antibiotic resistance, which occasionally occurs, is another matter because of the possibility of the resistance spreading to other, more harmful species and genera. New species and more specific strains of probiotic bacteria are constantly identified. Prior to incorporating new strains into products their efficacy should be carefully assessed, and a case by case evaluation as to whether they share the safety status of traditional food-grade organisms should be made. LAB may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens. The main threat associated with these bacteria is that they can transfer resistance genes to pathogenic bacteria. A number of initiatives have been recently launched by various organizations across the globe to address the biosafety concerns of starter cultures and probiotic microorganisms. This review can lead to better understanding of the role played by the dairy starter microorganisms in horizontal transfer of antibiotic resistance genes to intestinal microorganisms and dairy-biosafety.

***Keywords:*** Antibiotic resistance gene, starter culture, Plasmid, Probiotic bacteria.

## INTRODUCTION

Along with the metabolic activities of LAB commonly used as starters, they are required to be resistant to antibiotics and phage, Geis *et al.* (2003). Antibiotics which are used for the animals treatment, especially in mastitis therapy, pass into milk. Even at low concentrations of antibiotics resultaing in inhibition of acid development and poor quality dairy products can be noticed. Furthermore, they cause large economic losses, Kosikowski (1977). Many LAB species are involved in the manufacture and preservation of fermented feed and foods from raw agricultural materials (such as milk, meat, vegetables and cereals) in which they are present as contaminants or deliberately added as starters in order to control the fermentation process, having therefore a great economic importance. In addition, LAB contribute to the organoleptic, rheological and nutritional properties of fermented feed and foods, Leroy and de Vuyst (2004).

High sensitivity to most antibiotics employed in mastitis therapy has been reported both in *S. thermophilus* and *Lactobacillus* (*L.*) delbrueckii ssp. *bulgarcius* used for yoghurt production, Larsen and Anon (1989). Phenotypic assays have now been complemented by molecular methods in which bacterial strains are directly screened for the presence of antibiotic resistance determinants.

These methods include ampli.cation by PCR with speci.c primers for single or multiplex antibiotic resistance genes ,real time PCR or the use of DNA microarrays containing large collections of antibiotic resistance genes, Perreten *et al.* (2005).

Identification of LAB was done by different ways i.e. according to the physiological and morphological parameters by using Abi or Bergeys manual. The intergenic spacer region (ISR) between 16S and 23S rRNA genes was tested as a tool for differencing the majority of lactic acid isolated from dairy products. Moreover, results obtained after nucleotide analysis show that the 16S-23S rDNA ISR sequences are very reately, in size and sequence among *Lactoccocus* species, Blatiotta *et al.* (2002).

Studies on LAB strains from commercial sources indicate that up to 80% of isolates of this species currently use as starter culture are plasmid free Herman, and Mckay, (1985) and Janzen *et al.* (1992). Moreover, when plasmids are present in these bacteria, they are found in small sizes (2-8 Kb).

Meanwheile, studies on the selection and dissemination of antibiotic resistances have focused mainly on clinically relevant bacterial species. More recently, it was speculated that food bacteria may act as reservoirs of antibiotic resistance genes, O'Sullivan and Klaenhammer (1993), Axelsson and Ahrne (1988). Therefore, food may be important vehicles of enormous amounts of living bacteria, with biotechnical use as starter cultures, into the human body. These may carry transferable antibiotic resistances, which might be transferred to commensal or pathogenic bacteria. The presence of transmissible antibiotic resistance markers in the evaluation of strains is thus an important safety criterion. Antibiotic resistance has been shown to have occurred rarely in bacteria collected before the antibiotic, Hughes and Datta (1983). Shortly after the introduction of each new antimicrobial compound, emergence of antimicrobial resistance is observed, Levy (1997). The magnitude of the problem is significantly increased by the possibility of bacteria to transfer resistance determinants horizontally and by the mounting increase in the use (over-use and misuse) of antibiotics, which has created an enormous selective pressure towards resistant bacteria, Levy (1992). Evolution of antibiotic resistant food borne pathogens has been amply documented in recent years, Teuber and

Perreten (2000) and White *et al.* (2002). Many investigators have speculated that communal bacteria may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens, Levy and Salyers (2002) and are thus very important in our understanding of how antibiotic resistance genes are maintained and spread through bacterial populations. The main threat associated with these bacteria is that they can transfer resistance genes to pathogenic bacteria. Most food-associated with LAB have acquired the 'Generally Regarded As Safe' (GRAS) status, the potential health risk, due to the transfer of antibiotic resistance genes from LAB reservoir strains to bacteria in the resident microflora of the human gastrointestinal tract and hence to pathogenic bacteria, has not been fully addressed.

Plasmids are widely distributed in the bacterial world. In LAB especially they present in the mesophilic lactococci. Plasmids encode several essential functions for dairy fermentation like lactose and protein metabolism, aroma and exopolysaccharide synthesis. Genes of bacteriophage defense system as well as for bacteriocin production and immunity are also located on lactococcal plasmids. Plasmids have played an important role in the development and application of techniques for the genetic manipulation of key industrial traits in food-grade LAB, Geis *et al.* (2003).

There is little information regarding the presence of antibiotic resistance genes on starter strains and their potential to transfer the resistance genes to pathogens. A number of initiatives have been recently launched across the globe to address the biosafety concerns starter cultures and probiotic microorganisms, Hassan El-Demerdash (2006).

Thus, the intention of the present study amid to review the distribution and origin of antibiotic resistance in LAB, and the potential mechanisms of transfer and their role in dairy starter culture and its probiotc properties.

**The antibiotic resistance condition:**

The clinical use of antibiotics has achieved a significant reduction in the morbidity and mortality associated with infectious diseases, their use has been extended to veterinary medicine, where they are employed as therapeutic agents, prophylactics and animal growth promoters, and to agriculture for the control of plant diseases, Levy and Marshall (2004). Indeed, a correlation between antibiotic and resistance has repeatedly been reported.Resistances may be inherent to a bacterial genus or species (natural or intrinsic resistance) and acquired, either through one or more sequential mutations or by the incorporation of new genes, Levy and Marshall (2004). Intrinsic and acquired resistance by mutation are presumed to present in a low risk of horizontal spread, while the risk of transfer is maximal if acquired resistance is mediated by added genes, Normark and Normark (2002).Resistances are not virulence factors by themselves, but infections with resistant microorganisms complicate the course of the diseases and put up the value price of their treatment.

**Antibiotic resistance of dairy LAB:**

The knowledge of intrinsically coded resistance of LAB to common antibiotics is necessary to recognize acquired resistance traits. Enterococci are intrinsically resistant to cephalosporins and low levels of aminoglycoside and clindamycin, Teuber *et al.* (1999). *Lactobacilli*, *Pediococci* and *Leuconostoc* spp. have been reported to have a high natural resistance to vancomycin, a property that which is useful to separate them from other Gram-positive bacteria, Simpson *et al.* (1988). In study shows the resistance against antibiotics of *E. faecium* isolated from Egyption white cheese (Table 1). Data indicated that, all strains were sensitive to Vancomycin except *E. faecium* HM1, while the same strain was sensitive for all the other antibiotic, Amal *et al.* (2006).

Some lactobacilli have a high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim/sulphamethoxazole, and vancomycin, Danielsen and Wind (2003). For a number of lactobacilli a very high frequency of spontaneous mutation to nitrofurazone, kanamycin and streptomycin was found, Curragh and Collins (1992). From these data it is clear that intergenus and interspecies differences exist, and consequently identification at species level is required in order to interpret phenotypic susceptibility data.

Most of *S. thermophilus* strains were resistant to ampicillin (72.9%), gentamicin (70.8%) and streptomycin (68.7%) and susceptible to vancomycin (89.5%), tetracycline (81.2) and chloramphenicol (77.0%) and Plasmid DNA was detected in 29 strains, Hassan El-Demerdash (2006). Another study was undertaken to establish the levels of susceptibility of *Lactobacillus* spp. to various antimicrobial agents, Danielsen and Wind (2003) and it was shown to be species-dependant. For the following antimicrobial agents, susceptibility varied several folds between species: vancomycin, teicoplanin, tetracycline, norfloxacin, ciprofloxacin, fusidic acid, and clindamycin. The differences between the species were more subtle for the rest of the tested antimicrobials.

In a study undertaken by, Temmerman *et al.* (2002), a total of 55 European probiotic products were evaluated with regard to the identity and the antibiotic resistance of the bacterial isolates recovered from these products. Using the disc diffusion method, antibiotic resistance among 187 isolates was detected against kanamycin (79% of the isolates), vancomycin (65%), tetracycline (26%), penicillin G (23%), erythromycin (16%) and chloramphenicol (11%). Overall 68.4% of the isolates showed resistance against multiple antibiotics including intrinsic resistances. Investigated strains of *Lactococcus lactis* were sensitive to amikacin, ampicillin, 1st generation of cephalosporin, chloramphenicol, erythromycin, gentamicin, imipenem, oxacillin, penicillin, pipericillin, sulphonamide, tetracycline, trimethoprim/sulfomethoxazole, and vancomycin, de Fabrizio *et al.* (1994). A slightly lowered susceptibility was observed towards

carbenicillin, ciprofloxacin, dicloxacillin and norfloxacin. Intrinsic resistances were recorded towards colistin, fosfomycin, pipemidic acid and rifamycin. Recently, multiple drug efflux proteins were discovered in *Lc. lactis* subsp. *lactis* MG1363, van Veen and Konings (1998), one being an ABC transporter (*lmr* A), and the other proton motive force dependent drug transporter (*lmr* P). Both are responsible for a resistance to high concentrations of ethidium bromide. The natural substrates are not known. Twenty-six strains of *Lc. lactis* subsp. *cremoris* and subsp. *lactis* were all resistant to trimethoprim and almost all to sulfathiazole. Resistances to gentamicin, kanamycin, lincomycin, nafcillin, neomycin, nisin, rifampin and streptomycin varied, Orberg and Sandine (1985).

Thirty-one strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* as components of yoghurt cultures showed intrinsic resistance towards mycostatin, nalidixic acid, neomycin, polymyxin B, trimethoprim, colimycin, sufamethoxazol and sulphonamides. Susceptibilities to cloxacillin, dihydrostreptomycin, doxcycline, furadantin, novobiocin, oleandomycin, oxacillin and streptomycin were prominent while kanamycin and streptomycin susceptibilities varied, Sozzi and Smiley (1980). Many strains of *Lb. plantarum*, *Lb. casei*, *Lactobacillus salivarius*, *Lactobacillus leishmannii*, *Lb. acidophilus* carry intrinsic resistance towards vancomycin which is due to the presence of D-alanine: D-alanin ligase-related enzymes, Elisha and Courvalin (1995). 34 *S. thermophilus* strains isolated from Turkish yoghurts were examined for their antibiotic resistance patterns and plasmid carriage. Most strains of *S. thermophilus* were found to be resistant to gentamicin (79%) and penicillin G (64%) and susceptible to chloramphenicol (94%) and tetracycline (88%); however, no correlation was observed between the resistance to antibiotics and the occurrence of plasmids in some strains, Aslim and Beyatli (2004).

***Antibiotic resistance gene of LAB:***

A prerequisite to acquire antibiotic resistance genes from other bacteria is the potential of LAB to communicate actively and passively with these bacteria with the aid of conjugative plasmids and transposons. Plasmids are common in LAB, and differences are found in size, function and distribution, Wang and Lee (1997). The functions found on plasmids include hydrolysis of proteins, metabolism of carbohydrates, amino acids and citrate, production of bacteriocins and exopolysaccharides, and resistance to antibiotics, heavy metals and phages. Plasmids are common in enterococci, lactococci, leuconostocs, pediococci, and present in some strains of lactobacilli and bifidobacteria, Simpson and Taguchi, (1995) and Teuber (1995). Although several *S. thermophilus* plasmids have been sequenced, Janzen *et al.* (1992) and Geis *et al.* (2003), only recently, a few genes other than those necessary for plasmid replication have been identified, Geis *et al.* (1999). Certain conjugative antibiotic resistance plasmids, such as pAMβ1 and PIP501, are capable of both mobilization and intergeneric conjugation, Langella *et al.* (1993). Conjugative transposons (broad and narrow host rage) have been described in enterococci, lactococci and streptococci, Clewell, (1993).

***Plasmids encoding antibiotic resistance gene in LAB:***

Studies on LAB strains from commercial sources indicate that up to 80% of isolates of this species currently use as starter culture are plasmid free, Herman and Mckay, (1985), Janzen, *et al.* (1992).When plasmids are present in these bacteria, they are found in small sizes (2-8 Kb).

Characterizing the biochemical functions of plasmids and developing gene transfer systems for LAB and plasmid distribution patterns had been studied earlier. Most *S. thermophilus* strains were found to carry a limited number of plasmids, Somokuti, and Steinberg (1986). The role of many plasmids in LAB is unclear, since no obvious phenotypic trait has been associated with the presence of the plasmids. Plasmids have played an important role in the development and application of techniques for the genetic manipulation of key industrial traits in food-grade LAB. For this reason, it is interest to examine the sequence of native LAB plasmids with a view to exploring their application as food-grade vectors, and to determine the biological role of LAB plasmid, El Demerdash *et al.* (2003).

The only way to study the biological function of plasmids is to compare wild-type cells with plasmid-cured derivatives. Among several techniques for curing, protoplast formation and regeneration have gained importance for lactic streptococci.

LAB often harbor plasmids of different sizes, and some antibiotic resistance determinants located on plasmids have been reported to occur in *Lactococcus lactis* and various *Lactobacillus* and *Enterococcus* species, Gevers *et al.* (2003). Among the LAB, antibiotic resistance of the enterococci has been subject to intense study, Landman and Quale (1997) and Leclercq (1997), particularly because strains of these bacteria cause numerous and serious infections in humans, Morrison *et al.* (1997) and Murray (1990).

Several antibiotic resistance plasmids from lactobacilli have been detected. Ishiwa and Iwata (1980) indicated it by curing experiments the plasmid-linkage of tetracycline and erythromycin resistances in *Lactobacillus fermentum*. Curing techniques have been applied by study the strain dependent resistance to macrolides, tetracycline and chloramphenicol in *Lactobacillus acidophilus* and *Lactobacillus reuteri* of animal origin. Plasmids encoding tetracycline, erythromycin, chloramphenicol, or macrolide-lincomycin-streptogramin resistance have been reported in *S. thermophilus*, Plasmid DNA was detected in 29 strains, Hassan El Demerdsah (2006). The sizes of plasmid DNAs ranged from 2.3 to 7.9 Kb (Fig 1). A correlation was observed between the present of the plasmid and the resistance to the antibiotics. No clear correlation was observed between the size and the number of the plasmids with the resistance to the antibiotics (Table 2). However, it is not clear to construct correlation between some plasmids harbouring strains and their resistance to the antibiotics, *Enterococcus fecium*, Amal *et al.* (2006) (Fig 2), *Lb.*

*acidophilus*, Vescovo *et al.* (1982), and *Lb. plantarum*, Danielsen (2002), isolated from raw dairy products, and other fermented foods.

Most of these R-plasmids had a size smaller than 10 kb (5.7-18 kb). *Lb. fermentum* isolated from pig faeces carried a 5.7 kb plasmid with an *erm* gene conferring high level erythromycin resistance which was 98.2% identical to the gene of the enterococcal conjugative transposon Tn1545, Fons *et al.* (1997). The reported prevalence of antibiotic resistance genes such as erythromycin, vancomycin, tetracycline, chloramphenicol, and gentamicin resistance genes, on transferable genetic elements in enterococci is more extensive, both on plasmids, West and Warner (1985) and Clewell *et al.* (1974) and transposons, Perreten *et al.* (1997*b*). Plasmid could either be electrotransformed into other hosts or conjugatively transferred to a number of other Gram-positive bacteria by the help of a wide host range, originally enterococcal erythromycin resistance plasmid pAMß, Clewell *et al.* (1974).

Multiple antibiotic resistance plasmid pK214 was reported in a *Lc. lactis* strain K214 isolated from raw milk soft cheese, Perreten *et al.* (1997*a*), encoding streptomycin, tetracycline and chloramphenicol, and drug efflux gene mef 214. The tetracycline-resistant gene (providing ribosome protection) is 99.8% homologous to tet (S) from Listeria monocytogenes. Characterization of *mef* 214 demonstrated that it mediated multiple antibiotic resistance hence recently it has been renamed *mdt* (A) for multiple drug transporter. In *Lc. lactis* the gene mediated increased resistance to erythromycin and tetracycline, Perreten *et al.* (2001). Two other multidrug transporters have been described in *Lc. lactis*. The first (LmrA) is a member of the ABC superfamily; the second (LmrP) is a proton motive force dependent transporter, Bolhuis *et al.* (1995). Interestingly, multidrug transporter LmrA in *Lc. lactis* is a homologue of the human multidrug-resistance P-glycoprotein, another drug pump involved in drug resistance of human cancer cells encoded by the *MDR1* gene.

The presence of potentially transferable resistance genes in many LAB species is well.The nucleotide sequence identity of most determinants encountered in LAB and bifidobacteria to genes firstly described in distinct bacterial groups suggests that resistances emerged in microorganisms other than LAB and bifidobacteria, to which they were somehow transferred.

Plasmid encoding tetracycline resistance gene *tet* (M) was detected in *Lactobacillus* isolates from fermented dry sausages, Gevers *et al.* (2003). Conjugative transposons are a main type of vehicle regarding antibiotic resistance transport in Gram-positive bacteria.

## CONCLUSIONS

The systematic studies to investigate acquired antibiotic resistance of LAB in food origin are very important. Most data exist on opportunistic pathogenic enterococci, while the number of reports on lactococci and lactobacilli is limited. However, it is recently expanding due to increased interest in probiotic lactic acid bacteria and genetic modification of LAB for different purposes. The following general observations can be made after examination of these data.

There may be intrinsic resistance traits, e.g. vancomycin resistance in *Leuconostoc*, certain lactobacilli and others, or resistance to nalidixic acid. Distinction between intrinsic and acquired resistance is difficult as it is not possible to trace an investigated strain into the preantibiotic era. If LAB lives in a biotope challenged regularly with antibiotics (human intestine, animal intestine, bovine udder) acquired antibiotic resistance is found in LAB from such habitats including *Enterococcus*, *Lactococcus* and *Lactobacillus* species.

A correlation was observed between the present of the plasmid and the resistance to the antibiotics. No clear correlation was observed between the size and the number of the plasmids and the resistance to the antibiotics. Most of the wild type isolates of LAB carried low molecule weight plasmid DNA. Plasmid DNA of these strains may be used in recombinant DNA technology. Strains that showed resistance to most of the antibiotics, low acidity and bile salt tested may be also are used in the present of special conditions in the industrial process. Most of the results suggest that, LAB and other starter culture strains naturally resistance to antibiotics is favorable for use as probiotics or starter culture.

LAB, like all other bacteria are prone to gene exchange to enhance survival in antibiotic containing environments. For the food microbiologist, there is no doubt that it is necessary to avoid the distribution of bacteria with mobilizable antibiotic resistances. Measures include the use of proper starter cultures and proper substrates for food fermentations. For example, the transfer of antibiotic resistant bacteria from animals into fermented and other foods can be avoided if the raw substrate milk or meat is pasteurized or heat-treated. In addition, generation of antibiotic resistant bacteria in food animals and plants has to be minimized by prudent use of antibiotics. To preserve the life saving potential of antibiotics the spread of resistance genes at all levels must be stopped. Although special purpose probiotics for use in combination with antibiotics have been developed through the introduction of multiple resistances to the bacteria, probiotics generally should not be designed to carry more resistance than is required for a specific purpose. Above all the biosafety of the probiotic lactic acid bacteria (e.g. lactobacilli, lactococci, enterococci, bifidobacteria) for human consumption must be assessed by proposing criteria, standards, guidelines and regulations on the one hand, and standardizing methodologies of premarketing biosafety testing and post marketing surveillance on the other hand.

**Table (1):** Resistance against some antibiotics of E. *faecium* isolated from traditional Egyptian dairy products (+: Resistance -: Sensitive). (Amal *et al.*, 2005).

| Antibiotic Concentration | Growth of *E. faecium* strains | | | | | |
|---|---|---|---|---|---|---|
| | HM1 | HM2 | HM3 | HM4 | HM5 | HM6 |
| Kanamycin (30µg/ml) | - | - | + | - | + | + |
| Streptomycin (30µg/ml) | - | - | + | - | + | - |
| Erthromycin (10µg/ml) | - | - | - | - | - | - |
| Teteracycline (30µg/ml) | - | - | - | - | + | - |
| Chloramphenicol (30µg/ml) | - | - | - | - | - | + |
| Ampicillin (30µg/ml) | - | - | - | - | - | + |
| Penicillin (10µg/ml) | - | - | - | - | - | - |
| Vancomycin (30µg/ml) | + | - | - | - | - | - |

**Table (2):** Antibiotic susceptibility of S. *thermophilus* isolates (n=48) from Egyptian fermented dairy product. (Hassan El Demerdash, 2006)

| Antibiotic | Concentration (µg/disc) | Susceptible | | Resistance | | Intermediate | |
|---|---|---|---|---|---|---|---|
| | | *No.* | *%.* | *No.* | *%* | *No.* | *%* |
| Amp | 10 | 13 | 27.0 | 35 | 72.9 | 0 | 0 |
| Pen G. | 10 | 15 | 31.2 | 31 | 64.5 | 2 | 4.1 |
| Chl | 30 | 37 | 77.0 | 5 | 10.4 | 6 | 12.5 |
| Ery | 15 | 20 | 41.6 | 24 | 50 | 4 | 8.3 |
| Tet | 30 | 39 | 81.2 | 7 | 14.5 | 2 | 4.1 |
| Van | 30 | 43 | 89.5 | 3 | 6.2 | 2 | 4.1 |
| Gen | 10 | 10 | 20.8 | 34 | 70.8 | 4 | 8.3 |
| Str | 10 | 12 | 25.0 | 33 | 68.7 | 3 | 6.2 |
| Rif | 30 | 33 | 68.7 | 12 | 25 | 3 | 6.2 |
| Kan | 30 | 27 | 56.2 | 21 | 43.7 | 0 | 0 |
| Neo | 10 | 28 | 58.3 | 19 | 39.5 | 1 | 2.0 |

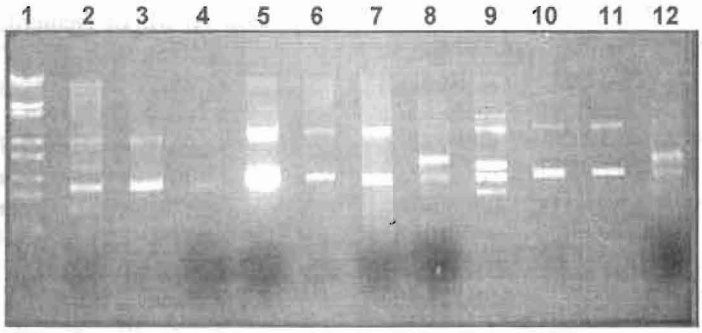


**Figure (1):** Plasmid profiles of *S. thermophilus* isolates
Lines: 1 DNA Marker ; Lines 2,3,4,5,6,7,8,9,10,11and 12 are different plasmid profile of *S. thermophilus* isolates (Hassan El Demerdash, 2006).

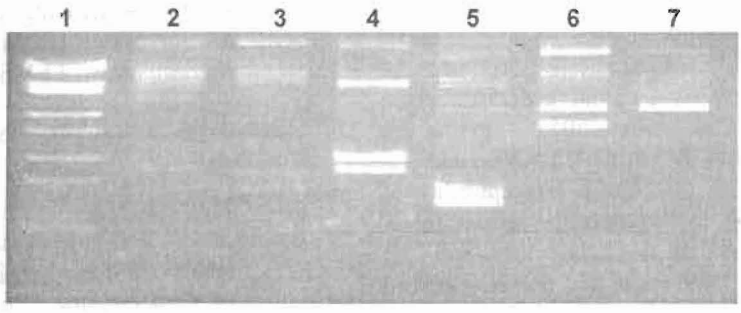


**Figure (2):** Plasmid profiles of isolated *E. faecium* strains
Lane 1: DNA marker; Lane 2,3,4,5 and 6 are: different Plasmid profiles of isolated *E. faecium* (Amal *et al.*, 2005).

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