Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 9 (6): 296-302, 2014
ISSN 1816-4935 / DOI: 10.3923/jm.2014.296.302

Antibiotic Resistance and Surviving Percentage of Lactic Acid Bacteria and *Bifidobacterium* spp.

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ABSTRACT

The outlines of antibiotic resistance of some probiotic microorganisms were studied. This study was conducted to evaluate the antibiotic resistance of LAB strains and *Bifidobacterium* spp., present in Egyptian markets. Disc diffusion method and optical density was used to determine the survival percentage of bacteria. After exposed to two antibiotics, 6 selected lactobacilli and bifidobacteria were assayed above were resistant *Lactobacillus lactis* to tetracycline (30 μg L^{-1}) but when these strains exposed to chloramephnicol (30 μg L^{-1}) we found that *L. acidophilus*, *L. casei*, *L. planterum* and *L. Lactis* were sensitive but *S. thermophilus* and *B. bifidum* were not. These strains were further investigated for their safety properties including sensitivity to antibiotic in MRS broth media the results showed that *L. lactis*, *L. acidophilus* and *B. bifidum* strains were the most resistant to tetracycline but *L. casei*, *S. thermophilus* and *B. bifidum* were the most resistant strains to chloramephnicol.

Key words: LAB, sensetivity, stability, antibiotics

INTRODUCTION

The balance and composition of the intestinal microbiotic is important for the well-being and the ability of our organism to resist the invasion of pathogens. To increase the natural resistance of the host to infections, probiotic microorganisms such as lactobacilli and bifidobacteria can be used.

Lactobacillus and *Bifidobacterium* species constitute a significant portion of probiotic cultures used in developed countries (Fuller, 1992). The actual safety criteria for successful probiotics have been defined in several reviews (Saarela *et al*., 2000). These criteria are as following: Strains for human use need to have a human origin and be isolated from healthy human gastrointestinal tract, they need to have a non-pathogenic history, not associated with diseases and do not carry transmissible antibiotic resistance genes.

Lactobacilli and *Bifidobacteria* have a long history of safe use as microbial adjunct nutrition (Salminen *et al*., 1998).

Lactic Acid Bacteria (LAB) form a taxonomically diverse group of microorganisms that can convert fermentable carbohydrates into lactic acids (Ammor *et al*., 2007). A large number of LAB species are involved in the production and consumption of fermented foods and beverages. Most LAB are omnipresent members of the intestinal flora. Bacteria in the human intestine play an important role in human physiology, most of which are beneficial or neutral for the host.

Antibiotic resistance can occur in two ways in a bacterial population: Mutation of an endogenous gene or acquisition of a resistance gene from an exogenous source. Mutations, which may cause genetic changes in multiple regions of the genome, play only a minor role in the development of resistance (Howden *et al*., 2006).

Although, the use of LAB has a long and safe history and has acquired the Generally Regarded as Safe (GRAS) status, the safety of selected strains should be evaluated before use, not only for virulence factors and other potential disease-causing traits, but also for their capability of acquiring and disseminating resistance determinants (Davies, 1994).

Lactic Acid Bacteria (LAB) comprise a wide range of genera and include a considerable number of species. These bacteria are the major component of the starters used in fermentation, especially for dairy products and some of them are also natural components of the gastrointestinal microflora. *Lactobacillus* is one of the most important genera of LAB (Coeuret *et al*., 2003).

During the last fifteen years, the *Lactobacillus* genus has evolved and contains to date more than 80 species. They are present in raw milk and dairy products such as cheeses, yoghurts and fermented milks (Coeuret *et al*., 2003). *Lactobacilli* comprise a large and diverse group of gram positive, nonspore forming, catalase negative rod bacteria, able to produce lactic acid as the main end-product of the fermentation of carbohydrates (Pelinescu *et al*., 2009).

The aim of present studies was to evaluate the antibiotic resistance of LAB strains present in Egyptian markets, in an attempt to contribute to the biosafety surveillance of LAB for human consumption.

MATERIALS AND METHODS

Antibiotics: The following 2 antimicrobial agents were used: Chloramphenicol and tetracycline. Each of the antibiotic powders was carefully weighed, dissolved, diluted in appropriate diluents and filter sterilized prior to addition to MRS broth medium. The following concentrations were tested: 25, 30 and 35 μg mL^{-1}.

Bacterial strains: The following 6 strains *L. plantarum*, *L. acidophilus*, *L. lactis*, *L. casei*, *B. bifidum* and *S. thermophilus* were obtained from MERCIN center, Faculty of Agriculture Ain Shams University.

Tetracycline and chloramephnicol sensitivity: The antibiotic susceptibility of selected two antibiotics, namely, tetracycline (25, 30 and 35 μg mL^{-1}), chloramphenicol (25, 30 and 35 μg mL^{-1}) was tested.

Disc diffusion method was used to screen for the antibiotic susceptibility of isolates with 12 discs (BD) containing chloramphenicol (C 30 μg mL^{-1}) and tetracycline (TE 30 μg mL^{-1}). Tests were conducted according to the criteria of the National Committee of Clinical Laboratory Standards (NCCLS) MRS agar for *Lactococci* and *Streptococci*, *Lactobacilli* and *Bifidobacteri*.

Inhibition-zone diameters were measured after incubation at 37°C for 4 and 24 h, as previously described (Charteris *et al*., 2001) and used as an indication for the borderline between susceptible and resistant isolates. Resistant strains were selected after compared with known standard.

The sensitivity of LAB and Bifidobacteria to tetracycline and chloramephnicol were evaluated in MRS supplemented with tetracycline and chloramephnicol using a modified method described by Pereira and Gibson (2002). Test *Lactobacilli* isolates cultures were grown for 4 and 24 h in MRS broth at 37°C.

An aliquot of 1 mL of the 4 h old culture was inoculated into 100 mL MRS broth with 25, 30 and 35 μg tetracycline and chloramephnicol (Sigma, USA).

Bacterial growth was monitored by determination of optical density at 640 nm after 4 and 24 h incubation period at 37°C. The percentage difference between the variation of Optical Density (OD) of culture without antibiotic (ΔOD0% antibiotic) and the variation of optical density of culture containing 25, 30 and 35 μg of antibiotics (ΔOD 25, 30 and 35 μg of antibiotics) would give an index of isolates surviving that can be expressed as follows:

$$\text{Surviving (\%)} = \frac{\Delta OD\neg 0\% \text{ antibiotics} - \Delta OD \text{ 25, 30 and 35 } \mu g \text{ of antibiotics}}{\Delta OD\neg 0\% \text{ antibiotics}} \times 100$$

Statistical analysis: One way analysis of variance (ANOVA) is applied on results.

RESULTS AND DISCUSSION

Antibiotic susceptibility: Antimicrobial disc-diffusion susceptibility of the 6 strains of tested lactic acid bacteria and bifidobacteria is summarized in Table 1. A total of 6 strains were resistant to tetracycline (30 μg mL^{-1}) but 2 of these strains were resistant to chloramephnicol (30 μg mL^{-1}).

Antibiotic sensitivity: After exposure to two antibiotics, 6 selected *Lactobacilli* and *Bifidobacteria* were assayed in Table 2. All strains demonstrated to resist antibiotics by presenting surviving percentage under exposure to 25, 30 and 35 μg mL^{-1} of each antibiotic after 4 and 24 h at 37°C. These strains were further investigated for their safety properties including sensitivity to antibiotic.

Table 2 showed that survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with tetracycline 25 μg mL^{-1} which explained that at concentration 25 μg mL^{-1} of tetracycline the most resistant strain was *L. lactis* (99.88%) after 4 h but the most sensitive was

Table 1: Diameters of the inhibition zones for 6 lactic acid bacterial strains in disc diffusion testing of 2 antimicrobial agents

	Inhibiton zone diameter range (mm)	
Species (strains)	Tetracycline (30 μg L^{-1})	Chloramephnicol (30 μg L^{-1})
L. acidophilus	ND	3.0
L. casei	ND	1.1
L. planterum	ND	2.1
L. lactis	ND	1.2
S. thermophilus	ND	ND
B. bifidum	ND	ND

Diameter of the disc is 6 mm, ND: Not detected

Table 2: Survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with tetracycline 25 μg mL^{-1}

	Control			25 (μg)			Survival percentage at 25 μg	
Strains	0 (h)	4 (h)	24 (h)	0 (h)	4 (h)	24 (h)	After 4 h	After 24 h
L. plantarum	0.070	0.128	0.463	0.209	0.019	0.233	14.71	49.86
S. thermophilus	0.013	0.243	0.416	0.174	0.045	0.164	18.27	39.00
L. casei	0.044	0.105	0.383	0.057	0.103	0.224	97.99	58.10
L. lactis	0.172	0.114	0.425	0.135	0.114	0.344	99.88	80.51
L. acidophilus	0.203	0.143	0.453	0.186	0.086	0.353	59.99	77.47
Bifidobacteria	0.244	0.163	0.493	0.204	0.103	0.234	63.02	46.97

Table 3: Survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with tetracycline 30 μg mL^{-1}

	Control			30 μg			Survival percentage at 30 μg	
Strains	0 h	4 h	24 h	0 h	4 h	24 h	After 4 h	After 24 h
L. plantarum	0.070	0.128	0.463	0.203	0.105	0.292	81.90	62.60
S. thermophilus	0.013	0.243	0.416	0.142	0.044	0.125	17.86	29.63
L. casei	0.044	0.105	0.383	0.161	0.012	0.133	11.32	34.34
L. lactis	0.172	0.114	0.425	0.185	0.016	0.344	13.92	80.51
L. acidophilus	0.203	0.143	0.453	0.182	0.032	0.305	22.23	66.87
Bifidobacteria	0.244	0.163	0.493	0.196	0.046	0.433	28.05	87.33

Table 4: Survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with tetracycline 35 μg mL^{-1}

	Control			35 μg			Survival percentage at 35 μg	
Strains	0 h	4 h	24 h	0 h	4 h	24 h	After 4 h	After 24 h
L. plantarum	0.070	0.128	0.463	0.093	0.034	0.233	26.43	49.86
S. thermophilus	0.013	0.243	0.416	0.145	0.053	0.114	21.56	26.98
L. casei	0.044	0.105	0.383	0.213	0.034	0.127	32.27	32.77
L. lactis	0.172	0.114	0.425	0.164	0.034	0.366	29.71	85.69
L. acidophilus	0.203	0.143	0.453	0.206	0.114	0.348	79.57	76.36
Bifidobacteria	0.244	0.163	0.493	0.223	0.086	0.344	52.59	69.28

Table 5: Survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with chloramephnicol 25 μg mL^{-1}

	Control			25 μg			Survival percentage at 25 μg	
Strains	0 h	4 h	24 h	0 h	4 h	24 h	After 4 h	After 24 h
L. plantarum	0.063	0.143	0.392	0.151	0.141	0.367	98.45	93.23
S. thermophilus	0.013	0.210	0.414	0.019	0.043	0.063	20.26	14.80
L. casei	0.023	0.114	0.305	0.064	0.075	0.133	65.67	43.30
L. lactis	0.123	0.111	0.304	0.124	0.106	0.082	95.38	26.66
L. acidophilus	0.143	0.222	0.343	0.036	0.210	0.311	94.37	90.32
Bifidobacteria	0.175	0.252	0.495	0.105	0.244	0.205	96.57	40.91

L. plantarum (14.71%). Where after 24 h at concentration 25 μg mL^{-1} of tetracycline the most resistant strain was *L. lactis* (80.51%) after 4 h but the most sensitive was *S. thermophilus* (39.00%).

Table 3 showed that survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with tetracycline 30 μg mL^{-1} which explained that at concentration 30 μg mL^{-1} tetracycline the most resistant strain was *L. plantarum* (81.90%) but the most sensitive strain after 4 h was *L. casei* (11.32%). Where after 24 h the most resistant strain was *L. lactis* (80.51%) but the most sensitive was *S. theremophilus* (29.36%). Devirgiliis *et al.* (2010) reported that *L. lactis* in Italian dairy products was resistant to tetracycline and also Gevers *et al.* (2003) reported that *Lactobacillus* spp. were resistant to tetracycline gentamicin 79% in fermented dry sausages.

Table 4 showed that survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with tetracycline 30 μg mL^{-1} which explained that at concentration 35 μg mL^{-1} tetracycline after 4 h the most resistant strain was *L. acidophilus* (79.57%). But the most sensitive strain was *S. theremophilus* (21.56%) where after 24 h found that the most resistant strain was *L. lactis* (85.69%) but the most sensitive strain was *S. theremophilus* (26.98%).

Table 5 showed that survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with chloramephnicol 25 μg mL^{-1} which explained that at concentration

Table 6: Survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with chloramephnicol 30 μg mL^{-1}

	Control			30 μg			Survival percentage at 30 μg	
Strains	0 h	4 h	24 h	0 h	4 h	24 h	After 4 h	After 24 h
L. plantarum	0.063	0.143	0.392	0.201	0.021	0.044	14.54	10.83
S. thermophilus	0.013	0.210	0.414	0.162	0.107	0.105	50.74	24.94
L. casei	0.023	0.114	0.305	0.162	0.073	0.160	63.92	52.15
L. lactis	0.123	0.111	0.304	0.175	0.084	0.101	75.56	32.91
L. acidophilus	0.143	0.222	0.343	0.074	0.014	0.007	6.08	1.69
Bifidobacteria	0.175	0.252	0.495	0.102	0.195	0.143	77.12	28.39

Table 7: Survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with chloramephnicol 35 μg mL^{-1}

	Control			35 μg			Survival percentage at 35 μg	
Strains	0 h	4 h	24 h	0 h	4 h	24 h	After 4 h	After 24 h
L. plantarum	0.063	0.143	0.392	0.166	0.024	0.023	16.64	5.47
S. thermophilus	0.013	0.210	0.414	0.141	0.114	0.126	54.07	30.02
L. casei	0.023	0.114	0.305	0.182	0.063	0.163	55.14	53.13
L. lactis	0.123	0.111	0.304	0.081	0.084	0.063	75.56	20.41
L. acidophilus	0.143	0.222	0.343	0.035	0.044	0.003	19.59	0.53
Bifidobacteria	0.175	0.252	0.495	0.074	0.196	0.156	77.52	31.02

25 μg mL^{-1} chloramephnicol after 4 h the most resistant strain was *L. plantarum* (98.45%) but the most sensitive strain was *S. thermophilus* (20.26%) where after 24 h found that the most resistant strain was *L. plantarum* (93.23%) but the most sensitive strain was *S. thermophilus* (14.80%).

Table 6 showed that survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with chloramephnicol 30 μg mL^{-1} which explained that at concentration 30 μg mL^{-1} chloramephnicol after 4 h the most resistant strain was *Bifidobacteria* (77.12%) but the most sensitive strain was *L. acidophilus* (6.08%). Temmerman *et al.* (2002) found that *L. acidophilus* was resistant to tetracycline 26% in European probiotic products and sensitive to chloramphenicol 11% in the same products. Where after 24 h found that the most resistant strain was *L. casei* (52.15%) but the most sensitive strain was *L. acidophilus* (1.69%).

Table 7 showed that survival (OD) of selected LAB and *Bifidobacteria* after 4 and 24 h in MRS broth media spiked with chloramephnicol 35 μg mL^{-1} which explained that at concentration 35 μg mL^{-1} chloramephnicol after 4 h the most resistant strain was *Bifidobacteria* (77.52%) but the most sensitive strain was *L. plantarum* (16.64%) where after 24 h the most resistant strain was *L. casei* (53.13%), but the most sensitive strain after 24 h of incubation was *L. acidophilus* (0.53%).

DISCUSSION

D'Aimmo *et al.* (2007) reported that MIC values of tetracycline that inhibit 50% of the strains belonging to the *L. acidophilus*, *L. casei*, *L. bulgaricus* and *S. thermophilus* were 32, 4, 16, 16 and 1 μg mL^{-1}, respectively. Where they reported that MIC values of tetracycline that inhibit 90% of the strains belonging to the *L. acidophilus*, *L. casei*, *L. bulgaricus* and *S. thermophilus* were 32, 4, 16, 16 and 0.5 μg mL^{-1}, respectively. These results agree with Pan *et al.* (2011) who reported that *S. thermophilus* is resistant to tetracycline.

D'Aimmo *et al.* (2007) reported that MIC values of chloramephnicol that inhibit 50% of the strains belonging to the *L. acidophilus*, *L. casei*, *L. bulgaricus* and *S. thermophilus* were 2, 8, 16, 16 and 8 μg mL^{-1}, respectively. Where they reported that MIC values of tetracycline that inhibit 90% of the strains belonging to the *L. acidophilus*, *L. casei*, *L. bulgaricus* and *S. thermophilus* were 2, 8, 16, 16 and 4 μg mL^{-1}, respectively.

A single bacterial strain may possess several types of resistance mechanisms. The resistance mechanism categorized as biochemical and genetic types. Among these, which mechanism prevails in the specific bacterial strain depends on the nature of the antibiotic, its target site, the bacterial species itself and whether it is related by a plasmid or by a chromosomal mutation. Target bypass-some bacteria become refractory to specific antibiotics by bypassing the inactivation of a given enzyme (Dzidic *et al.*, 2008).

Most of these mechanisms have been observed and studied in various bacteria, however, there have not been specific studies dealing with these mechanisms in LAB or *Bifidobacteria*.

ACKNOWLEDGMENT

The study was financially supported by the Laboratories of Dairy Science Department and Food Safety and Quality Control Lab at Faculty of Agriculture Cairo. They fully agree to the publication of the data.

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