

Antibiotic Sensitivity of *Bacillus clausii* Strains in Commercial Preparation

A. Abbrescia¹, L.L. Palese¹, S. Papa², A. Gaballo³, P. Alifano⁴ and Anna M. Sardanelli^{1,5*}

¹Department of Basic Medical Sciences Neurosciences and Sense Organs, University of Bari Aldo Moro, Italy; ²Institute of Biomembranes and Bioenergetics (IBBE), Italian Research Council, (CNR), Bari, Italy; ³Institute of Nanoscience-NNL, Italian Research Council, (CNR), Lecce, Italy; ⁴Department of Biological and Environmental Sciences and Technologies (DiSTeBA), University of Salento, Lecce 73100, Italy; ⁵Institute of Translational Pharmacology (IFT) Italian Research Council, (CNR) Roma

Abstract: Recently has been acknowledged the healthy use of *Bacillus* and related bacteria as probiotics. A mixture reported to contain four probiotic strains of *Bacillus clausii* is marketed as an OTC (Over The Counter) medicinal supplement for human use. Their poliantibiotic resistant property, useful for restoring the gut microbiota during antibiotic treatment, raises the question about the risk of resistance transfer. In order to better assess the risk-benefit ratio it is important to always monitoring the pattern and stability of resistance spectra in these bacteria. In this work, we have extensively redefined the antibiotic susceptibility profile of these four probiotic strains. Resistance phenotype has been determined by screening a large number of antibiotics, including natural products (such as penicillin, vancomycin and erythromycin), and completely synthetic molecules (such as fluoroquinolones). Extensive comparison with a wild type strain belonging to the normal intestinal microbiome was carried out. The molecular basis of some resistances was determined. Observed antibiotic resistances were correlated with previous and new data in safety evaluations of these strains for human use.

Keywords: Antibiotic resistance, Beneficial microbes, *Bacillus clausii*, Probiotics.

INTRODUCTION

The healthy use of *Bacillus* and related bacteria as probiotics in humans has been acknowledged during the last years [1, 2]. Moreover, it has been suggested that the spore bearing bacilli have some potential advantages over other non-spore formers such as *Lactobacillus* spp., since spores are heat-stable, capable of surviving the low pH of the gastric barrier and products made on them can be stored at room temperature without any deleterious effect on viability [3, 4]. *Bacillus clausii* is a gram-positive, aerobic, endospore-forming,

facultative alkaliphilic rod bacterium, used as a human probiotic [5, 6]. The role of *B. clausii* in prevention and treatment of acute intestinal infection [7], prevention of side effects due to antibiotic therapy [8], stimulation of systemic immunoglobulin regulation, and in antimicrobial activity against Gram-positive bacteria [9, 10], has been recognized. In addition, the ability of *B. clausii* spores to germinate in experimental conditions mimicking the gastrointestinal tract is consistent with the beneficial health effects reported for this spore-forming bacterium [11]. The latest evidence locating *B. clausii* species not only in the chicken gut but also in the human gut, proves that these spore-formers have the potential to persist in (or transiently associate with) the complex gut ecosystem [12].

*Address correspondence to this author at the Department of Basic Medical Sciences Neurosciences and Sense Organs, Piazza G. Cesare, 11 70124, Bari, Italy; Tel: +390805448533; Fax: +390805448538; E-mail: annamaria.sardanelli@uniba.it

A spore mixture of four bacterial strains of *B. clausii*, known as O/C, SIN, N/R and T, characterized by an extended pattern of resistance to many antibiotics [13], are marketed in Italy as an OTC medicinal supplement.

These strains recently characterized at bioenergetic and proteomic level [14-16] are phenotypically distinguished by specific levels of resistances to chloramphenicol, streptomycin, rifampicin and tetracycline [8]. Frequently, microorganisms in commercially available probiotics have been shown to carry some antibiotic resistances [17], therefore these probiotic preparations are frequently used in the clinical practice during antibiotic therapies in order to prevent intestinal microbial imbalance. Nevertheless, the purported benefit is in contrast to the safety criteria, which state that, in order to decrease the concern over the antibiotic resistance transfer and spreading within the gastrointestinal tract from probiotics to pathogens, bacteria for human consumption should not carry any transferable antimicrobial resistance genes [18].

The hazard extent differs when dealing bacteria with: (i) intrinsic (natural phenotypic traits) or (ii) acquired resistances (particularly through mobile genetic elements, such as plasmids and transposons). Resistance by mutation in chromosomal genes represents always the lower risk of dissemination [19].

Thus, to better assess the risk-benefit ratio, it is important to always monitoring the pattern and stability of resistance spectra in the probiotics, in order to better manage the advantage of antibiotic-resistant probiotics during antibiotic therapy.

In this work we have extensively redefined the antibiotic sensitivity pattern of these four probiotic strains, since the more comprehensive data on this topic arise from studies dating back years [8, 13]. The strains O/C, SIN, N/R and T were compared with the type strain *B. clausii* DSM8716. Resistance phenotype has been determined by the screening against a large number of antibiotics, including natural products (such as penicillin, vancomycin and erythromycin), and completely synthetic molecules (such as fluoroquinolones). All major pharmacological classes have been considered in the screening, including drugs marketed for decades, as well as several antibiotics that have been only recently clinically approved.

Standard molecular techniques, such as PCR and direct sequencing, have been used to screen/identify mutations conferring specific individual traits of resistance.

MATERIALS AND METHODS

Bacterial Strains and Antibiotic Susceptibility Assays

The four *B. clausii* strains O/C, SIN, N/R and T marketed in Italy as Enterogermina OTC medicinal, were obtained from Sanofi as separate spore suspension. *Bacillus subtilis* ATCC6633, *B. clausii* DSM8716 and *Escherichia coli* ATCC25922 were used as reference/control strains. Antibiotic susceptibility assays were performed using the Kirby-Bauer method by agar diffusion technique. This technique was applied both with the disc diffusion method, and with the gradient method E-test to measure the minimum inhibitory concentration (MIC). All tests followed the testing and quality assurance practices outlined by the European Committee on Antimicrobial Susceptibility Testing (Eucast) (<http://www.eucast.org>). Antibiotic discs and MICE evaluator strips were provided from Oxoid (UK).

Since there are not many official data for the sensitivity to antibiotics for *Bacillus* genus, characterization of the susceptible or resistant phenotype was made with reference to the judgments based on Eucast interpretative standards for gram-positive bacteria. All values obtained by these tests, *i.e.* the diameters of the inhibition zones and MIC values, were then compared with threshold values (breakpoints) set by Eucast for gram-positive bacteria.

PCR Amplification and DNA Sequencing

To investigate whether specifically observed rifampicin and streptomycin resistances were due to mutations in, respectively, the rifampicin resistance-determining regions (RRDR) of the *rpoB* gene and in the *rpsL* gene, these encoding regions were PCR amplified. The custom-designed primers for the *rpoB* gene were 5'-CGCATTGGAGAAAA-TGT-3' (forward) and 5'-AAAACGGAATACATGACGTCG-3' (reverse), and the primers for the *rpsL* gene were 5'-AACCAGTTAATCCGCAAAGG-3' (forward) and 5'-GGTTTTGAACACCAGCGTG-3' (re-

verse). Amplification conditions for both the *rpoB* and the *rpsL* genes consisted of 30 cycles of 95°C for 45 s, 55°C for 1 45 s, and 72°C for 1 min. The amplification reactions were carried out in a Perkin–Elmer Cetus DNA cyclor 480. The resulting PCR products were sequenced bidirectionally by cycle sequencing on an automated ABI 310 sequencer using the Big Dye Terminator kit according to the manufacturer’s instructions (PE Applied Biosystems). The deduced amino acid sequences were aligned with those retrieved from the GenBank database by using the CLUSTAL W (<http://www.ebi.ac.uk/tools/clustalw2>).

Statistical Analysis

Antibiotic resistance profiles were calculated for each strain as the average of three independent assays using three different culture, such as a total number of nine different measures have been obtained for each strain and antibiotic. These data were used to determine the strain relationships by cluster analysis. Data were arranged in an *m*-by-*n* matrix where *m* is the number of strains and *n* the number of tested antibiotics. Strain distance was calculated by taxicab metric: the distance d_{pq} between two row vector x_p and x_q was

$$d_{pq} = \sum_j |x_{pj} - x_{qj}| \quad \text{Eq. (1)}$$

with $j = 1, \dots, n$. Linkage analysis was performed by the nearest neighbor method. If n_r is the number of objects in cluster *r* and n_s is the number of objects in cluster *s*, the distance used for linkage analysis d_{rs} was

$$d_{rs} = \min(\text{dist}(x_{ri}, x_{sj})) \quad \text{Eq. (2)}$$

where $i = 1, \dots, n_r$ and $j = 1, \dots, n_s$.

RESULTS

Antibiotic Susceptibility Profile

The present experimental data provide an updated overview of the antibiotic susceptibility profile among the four probiotic strains and related *Bacillus* strains. Average values of inhibition halo diameter are shown in Table 1; the level of resistance was estimated by measuring the MIC of antibiotics most representative of each class and the obtained results are displayed in Table 2. Through this study, we found that all *B. clausii* strains were sensitive to: ampicillin, amoxicillin, piperacillin,

cephalexin, cephalosporin, cefaclor, cefprozil, carbapenemics, glycopeptides, quinupristin dalfopristin, linezolid, fluoroquinolones. Instead, they share similar phenotypic resistance pattern summarized in Table 3. Evident variability in susceptibility among *B. clausii* strains was observed testing the group of cephalosporins. Often, the sensitivity level to a specific agent was found to be different among the four probiotic strains both in terms of halo of inhibition diameter and MIC values (Table 1-2). In particular, all *B. clausii* strains were resistant against cefuroxime, ceftriaxone, and cefotaxime, using the MIC and zone diameter breakpoint values suggested by Eucast. The resistance against the cefepime, a fourth generation cephalosporin, is inferred by the close to zero extent of inhibition zone (Table 1).

All *B. clausii* strains exhibit the so-called MLS_B resistance phenotype [20]. Specifically, we found no inhibition in the presence of the macrolides tested except for telithromycin and for quinupristin-dalfopristin for which a slight inhibition was detected in the four probiotic strains. Inhibition zones relative to telithromycin fell below the susceptibility for gram-positive models, putting the four probiotic strains in the resistant category, while the reference strain DSM8716 resulted phenotypically not susceptible to this agent. All *B. clausii* strains, except for SIN strain, were sensitive to aminoglycosides (streptomycin, neomycin, gentamycin).

The resistance of SIN strain to aminoglycosides was previously explained by the presence of an aminoglycoside nucleotidyl transferase gene phenotypically expressed only in SIN [21]. Except for the resistant T strain, all *B. clausii* strains were sensitive to classic tetracyclines such as tetracycline, oxytetracycline, doxycycline, and minocycline. Moreover, all strains, without exception, are sensitive to tigecyclin that is representative of the new class of glycylcyclines. Regarding chloramphenicol, the four probiotic strains were resistant, with O/C strain being highly resistant and others with a lower resistance degree, while the reference strain *B. clausii* DSM8716 is very sensitive. This resistance trait, shared by the four probiotic strains, is due to the expression of a chloramphenicol acetyltransferase (CAT) encoded by the chromosomal *catBcl* gene [22].

Table 1. Inhibition zone diameter (mm) ^{a, b}.

Antibiotic	µg/disk	<i>B.clausii</i> O/C	<i>B.clausii</i> SIN	<i>B.clausii</i> N/R	<i>B.clausii</i> T	<i>B.clausii</i> DSM8716	<i>B.subtilis</i> ATCC6633
Amoxicillin	25	20±0.5	19±0.7	17±0.5	22±1.1	16±0.6	26±0.6
Oxacillin	5	8	0	0	9±1.1	0	26±1.1
Piperacillin	100	15±0.5	15±0.6	14±0.6	17±0.8	12±0.6	26±0.5
Cefalexin	30	28±0.8	28±0.5	30±0.5	31±0.6	25±0.6	33.3±2.4
Cefazolin	30	15±0.9	14±0.8	13±0.4	18±0.6	12±0.7	40±2
Cefaclor	30	33±1.3	29±1.5	33±1.5	36±2.4	28±0.8	39
Cefuroxime	30	10±0.7	0	0	12±0.8	0	24±0.6
Ceftriaxone	30	21±1.3	15±0.7	13±0.7	25±0.7	18±0.5	27±0.7
Cefotaxime	30	19±0.9	14±0.9	11±1.3	22±1.5	14±1.8	30
Cefprozil	30	30	25±1.2	30±1	35±0.8	26±0.5	39±1.4
Cefepime	30	8±1	0	0	11±0.5	0	27
Imipenem	10	29±1.3	29±1.9	30±2.3	36±0.5	28±0.6	40
Meropenem	10	24±0.5	23	25±0.8	28±1.3	22±0.8	37±1.4
Teicoplanin	30	20±0.5	22	21±0.5	21±0.5	20±0.5	18
Vancomycin	30	22±0.5	24±0.5	23±0.5	24±0.5	21±0.7	19±0.7
Streptomycin	300	28±0.4	0	26±0.6	30±0.5	25	26±0.7
Neomycin	30	24	12±0.5	24±0.5	26±0.5	23	20
Amikacin	30	27±1	19±0.6	28±1.2	31±1	25±0.6	25±1.4
Gentamicin	120	28±1.5	28±1.3	27±1	26±1.7	27	27±0.4
Erythromycin	30	0	0	0	0	0	29±0.8
Azithromycin	15	0	0	0	0	0	18±0.7
Clarithromycin	15	0	0	0	0	0	26±0.6
Spiramycin	100	0	0	0	0	0	20±0.9
Telithromycin	15	15±0.5	18±0.5	15±0.9	19±1.1	0	25
Clindamycin	10	0	0	0	0	0	12.7±0.5
Lincomycin	15	0	0	0	0	0	22±0.6
Quinupristin/Dalfopristin	15	18±0.7	20±0.7	20	20	14±0.7	18±0.7
Tetracycline	30	21±1	27±0.8	22±0.8	11±0.5	21±0.5	29±0.7
Oxytetracycline	30	22±0.5	28	23±0.5	12±0.5	22±0.7	26
Doxycycline	30	24	31±0.5	26±0.5	17±0.5	24±0.7	31
Minocycline	30	20±0.6	28±0.5	24	18±0.8	23	31
Tigecycline	15	26±0.6	27±0.6	27	24±0.5	25±0.7	25
Chloramphenicol	50	0	16±0.6	13	15±0.6	25±0.5	27±0.7
Linezolid	30	28±0.5	31±0.6	28±0.8	31	26±0.6	28±0.7
Rifampicin	30	24±0.5	26±0.5	0	27±0.6	34	21±0.7
Nalidixic acid	30	16±0.5	16	15±0.5	16	16	25±1.4

(Table 1) Contd....

Antibiotic	µg/disk	<i>B.clausii</i> O/C	<i>B.clausii</i> SIN	<i>B.clausii</i> N/R	<i>B.clausii</i> T	<i>B.clausii</i> DSM8716	<i>B.subtilis</i> ATCC6633
Ciprofloxacin	5	25±0.5	26	25	26±0.5	20±0.7	32±0.7
Levofloxacin	5	23±0.5	23	23	25±0.5	19	32
Norfloxacin	10	21±0.8	21±0.5	20±0.5	21±0.5	14±0.7	29±1.4
Moxifloxacin	5	26±1	27±0.5	26±0.5	28±0.5	20±0.7	31±2.1
Metronidazole	50	0	0	0	0	0	0

a - All values are reported as the means of $\geq 3 \pm$ SD experiments for each strain-antibiotic combination with a number of replica plates ≥ 5 for each test.

b - All values obtained using *E. coli* ATCC 25922 as quality control strain (not shown), are in acceptable ranges displayed in Eucast Quality Control Tables.

Table 2. Minimum inhibitory concentration (MIC) of selected antibiotics.

Antibiotic	(range µg/ml)	MIC (µg/ml)					<i>B.subtilis</i> ATCC6633
		<i>B.clausii</i> O/C	<i>B.clausii</i> SIN	<i>B.clausii</i> N/R	<i>B.clausii</i> T	<i>B.clausii</i> DSM8716	
Amoxicillin	(256-0.015)	0.12	1.5	2	0.12	2	0.03
Oxacillin	(256-0.015)	12	16	12	6	16	0.09
Penicillin G	(256-0.015)	1	3	2	1	4	0.02
Ceftriaxone	(32-0.002)	8	>32	>32	3	>32	0.5
Cefotaxime	(256-0.015)	4	24	32	3	16	0.25
Imipenem	(32-0.002)	0.25	0.25	0.18	0.12	0.18	0.015
Meropenem	(32-0.002)	0.37	0.5	0.37	0.25	0.5	0.03
Ciprofloxacin	(32-0.002)	0.25	0.25	0.25	0.25	0.5	0.03
Levofloxacin	(32-0.002)	0.25	0.25	0.25	0.25	1	0.05
Amikacin	(256-0.015)	0.03	2.5	0.03	<0.015	0.06	0.12
Teicoplanin	(256-0.015)	0.12	0.06	0.06	0.09	0.06	0.12
Vancomycin	(256-0.015)	0.12	0.12	0.12	0.12	0.12	0.12
Erythromycin	(256-0.015)	≥ 64	≥ 16	≥ 64	≥ 8	>64	0.06
Clindamycin	(256-0.015)	>32	32	>32	32	>32	0.5
Tetracycline	(256-0.015)	3	0.4	2	64	3	0.06
Tygecycline	(256-0.015)	<0.015	0.015	0.03	0.25	<0.03	0.03
Linezolid	(256-0.015)	1	0.25	1	0.7	1	0.7

Respect to rifampicin all *B. clausii* strains, except, as known, for the highly resistant N/R strain, exhibited fully susceptibility to this agent. The tests for fluoroquinolones (ciprofloxacin, levofloxacin, norfloxacin, moxifloxacin), showed that the four probiotic strains are sensitive to these antibiotics. Interestingly, the reference strain *B. clausii* DSM8716 was found resistant to norfloxacin and moxifloxacin.

Genetic Characterization of Streptomycin and Rifampicin Resistances

In addition to drugs inactivation by modifying enzymes, another well recognized mechanism of resistance to aminoglycoside is due to ribosomal alteration. Specifically, the streptomycin acts by binding to the bacterial ribosome and causing mistranslation of proteins. Spontaneous streptomycin resistance is often associated with muta-

Table 3. Resistance pattern in *Bacillus clausii* strains.

	O/C			SIN			N/R			T			DSM8716		
	H	MIC	I.C.	H	MIC	I.C.	H	MIC	I.C.	H	MIC	I.C.	H	MIC	I.C.
Penicillin G	-	1	R	-	3	R	-	2	R	-	1	R	-	4	R
Oxacillin	8	12	R*	0	16	R*	0	12	R*	9	6	R*	0	16	R*
Cefuroxime	10	-	R*	0	-	R*	0	-	R*	12	-	R*	0	-	R*
Ceftriaxone	21	8	R	15	32	R	13	>32	R	25	3	R	18	32	R
Cefotaxime	19	4	R	14	24	R	11	32	R	22	3	R	14	16	R
Cefepime	8	-	R*	0	-	R*	0	-	R*	11	-	R*	0	-	R*
Erythromycin	0	>64	R	0	>16	R	0	>64	R	0	>8	R	0	>64	R
Azithromycin	0	-	R*	0	-	R*	0	-	R*	0	-	R*	0	-	R*
Clarithromycin	0	-	R*	0	-	R*	0	-	R*	0	-	R*	0	-	R*
Spiramycin	0	-	R*	0	-	R*	0	-	R*	0	-	R*	0	-	R*
Telithromycin	15	-	R	18	-	R	15	-	R	19	-	R	0	-	R
Clindamycin	0	>32	R	0	32	R	0	>32	R	0	32	R	0	>32	R
Lincomycin	0	-	R*	0	-	R*	0	-	R*	0	-	R*	0	-	R*
Metronidazole	0	>256	R	0	>256	R	0	>256	R	0	>256	R	0	>256	R

R, resistant.

H, halo of inhibition diameter.

I.C., interpretative categories.

MIC, minimum inhibitory concentrations ($\mu\text{g/ml}$).

n.d., not determinable, breakpoints not available.

-, strip or disks not possible for purchase.

*, resistance inferred by no visible inhibition zone or inhibition zone close to zero.

tions in chromosomal genes encoding 16S rRNA (*rrs*) and ribosomal protein S12 (*rpsL*), which prevent the antibiotic from binding to the ribosome [23]. To investigate the basis of streptomycin resistance in the SIN strain, the *rpsL* loci were PCR amplified and sequenced in the four probiotic strains. Analysis of the multi alignment performed on the obtained sequences, shows the presence in SIN of a transition AAA - AGA, specifying the K101R mutation in *rpsL* (corresponding to K88R in *E. coli rpsL*) (Table 4). It can be stated that the observed high level of resistance to streptomycin in SIN, is associated with this chromosomal point mutation.

Rifampicin binds to the β subunit of the RNA polymerase encoded by *rpoB* and inhibits transcription. In gram-positive bacteria, resistance to rifampicin is due to mutation in the antibiotic molecular target of the and in more than 90% of cases, rifampin selection in prokaryotes leads to isolation of missense mutations, deletions or inser-

tions in the 81-bp rifampicin resistance-determining region (RRDR) of the *rpoB* gene [24]. This is one of the rare cases where a single mutation may be sufficient to confer high-level, clinically significant resistance upon an organism. To test this hypothesis, PCR reactions were performed using heterologous primers for *rpoB* gene on the genomic DNA of the four probiotic strains. The sequence analysis of about 900 bp, obtained in RRDR region, revealed presence only in the strain N/R of a transition TTT - TCT, which results in the S487F (*B. subtilis* numbering) missense related to a rif mutation shown in Table 4. Then the high resistance to rifampicin of N/R strain depends on the presence of this point mutation at chromosomal level.

Cluster Analysis of Antibiotic Sensitivity Patterns

Statistical analysis shows that antibiotic sensitivity pattern permits a clear classification of the

Table 4. Clustal W alignments of deduced amino acid sequences of *rpoB* and *rpsL* genes product ^{a,b}.

Sequence type and strain	Resistance phenotype	Partial <i>rpsL</i> or <i>rpoB</i> (RRDR) amino acid sequence
<i>rpsL</i>		
<i>B. clausii</i> O/C	S	HNLQEHSVVLIRGGRVKDLPGVRYHIVRGAL
<i>B. clausii</i> SIN	R	HNLQEHSVVLIRGGRV R DLSGVRYHIVRGAL
<i>B. clausii</i> N/R	S	HNLQEHSVVLIRGGRVKDLPGVRYHIVRGAL
<i>B. clausii</i> T	S	HNLQEHSVVLIRGGRVKDLPGVRYHIVRGAL
<i>rpoB</i>		
<i>B. clausii</i> O/C	S	GSSQLSQFMDQTNPLAELTHKRRLSAL
<i>B. clausii</i> SIN	S	GSSQLSQFMDQTNPLAELTHKRRLSAL
<i>B. clausii</i> N/R	R	GSSQLSQFMDQTNPLAELTHKRRL F AL
<i>B. clausii</i> T	S	GSSQLSQFMDQTNPLAELTHKRRLSAL

^a R, resistant; S, sensitive.

^b Substituted amino acids that result in a resistant phenotype are indicated in boldface.

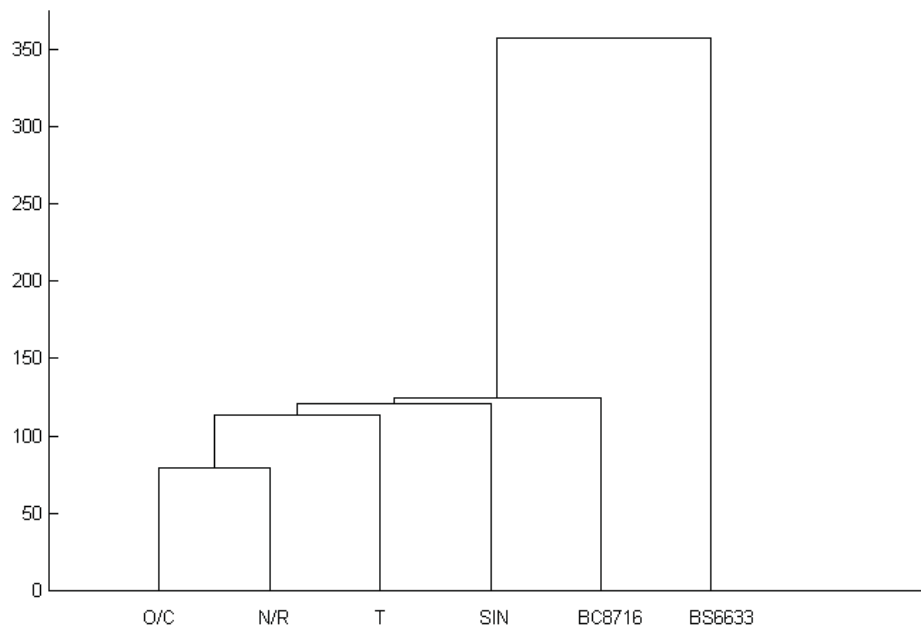


Fig. (1). Dendrogram of antibiotic resistance profiles for the *Bacillus* strains. Antibiotic resistance profiles were calculated for each strain as the average of three independent assays using three different culture, such as a total number of nine different measures have been obtained for each strain and antibiotic. Reported distances were determined by taxicab metric and nearest neighbor linkage analysis. For further details see under Methods.

analyzed strains (Fig. 1). Using an appropriate metric describing the strain distance, the *B. clausii* strains are grouped together and well separated from the *B. subtilis* strain, with the four probiotic strains more clustered respect to the reference strain. These results suggest that the antibiotic resistance pattern could be used also for classifica-

tion tasks, which is particularly difficult when closely related bacilli are considered.

DISCUSSION

The awareness that the complex communities of microbes that reside the human body play a critical role in the health of the host has led to a

number of researches [25-27]. One of the key functions that have been attributed to the gut microbiota concerns the metabolic functions carried out by this community [28]. The combined coding capacity of the microbiome as a metagenome, largely exceeds that of the host. The gut microbiota can metabolize nutrients ingested by the host as well as products of the hosts on metabolism. In turn, the host can further convert products of the microbial metabolism. Thus, the metabolite profile in the gut results from a combination of host and microbial metabolism. By this way, antibiotic administration can also have a deep effect on the metabolic profile of the host. Moreover, it is well known that immunologic interactions between the host and microbiota are intricate and important [25, 29].

This work is an upgrade of previous reports documenting the antibiotic sensitivity pattern of these marketed *B. clausii* strains [8] and provides an extensive phenotypic analysis of the antibiotic resistance potential of these strains. This is relevant for the general topic of safety of the microorganisms of human use, although antibiotic resistance per se is not a safety issue; it only becomes such when the risk of resistance transfer is present.

We observe that *B. clausii* and *B. subtilis* have species-specific sensitivity phenotypes, being *B. subtilis* widely susceptible to the antibiotics tested, while *B. clausii* strains are not.

We have found remarkably the similarity of phenotypic resistance patterns of four probiotic strains with the type strain DSM8716 (Table 3) and together, the five *B. clausii* strains share similar chromosomal genes involved in resistance to macrolides, beta-lactams and aminoglycosides [21, 20, 30]. In contrast, we found the type strain *B. clausii* DSM8716 fully sensitive to chloramphenicol, rifampicin, streptomycin and tetracycline, as indication that the strains O/C, SIN, N/R and T have acquired these resistances through mutagenesis selective process. While for the chloramphenicol resistance in four probiotic strains a chromosomal gene has been indicated [22], until now, no genetic data on the streptomycin, rifampicin and tetracycline resistances were available. Then, the analysis of the mutations involved in the resistance to streptomycin and rifampicin has revealed the K101R missense in *rpsL* gene, associated with the streptomycin resistance in SIN strain and the rif

mutation, S488F, in *rpoB* gene, associated with the rifampicin resistance in the N/R strain. Only for tetracycline, we could not provide any clue as to the mechanism of resistance, since the T strain does not contain sequences related to the "tet genes" [31] most commonly responsible for acquired tetracycline resistance in pathogenic bacteria (unpublished data).

In conclusion, similarly to several probiotic strains marketed in Europe [32], specific antibiotic resistance traits, both intrinsic and acquired by mutation of indigenous genes, were found in these four strains. Further verification of the lack of antibiotic resistance transfer in intestinal pathogenic bacteria through additional investigation on the genetic bases of some resistances traits are needed, because so far a clear resistance mechanism has not been identified and the presence of mobile genetic elements cannot be completely ruled out. But interestingly, recent findings show that *Bacillus* species, can be readily isolated from human faeces and frequently these include *B. clausii* strains with high similarity to the wild type strain DSM8716 [26, 27]. In this study, we highlighted the similarity of phenotypic resistance patterns of the four probiotic strains with the type strain DSM8716, and then it is reasonable to suggest that *B. clausii*, naturally bearing several resistance traits, is not uncommon in the natural commensal flora. Finally, our in deep characterization of the antibiotic resistance pattern of this widely used probiotic, together with the lack of significant iatrogenic issues despite several years of clinical use, can help a more insight full use of them in the clinical practice.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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REFERENCES

- [1] Hong HA, Duc le H, Cutting SM. The use of bacterial spore formers as probiotics. *FEMS Microbiol Rev* 2005; 29: 813-35.

- [2] Sanders ME, Morelli L, Tompkins TA. Spore formers as human probiotic: *Bacillus*, *Sporolactobacillus* and *Brevibacillus*. *Comp Re Food Sci Food Safe* 2003; 2: 101-10.
- [3] Cutting SM. *Bacillus* probiotics. *Food Microbiol* 2011; 28: 214-220.
- [4] Spinosa MR, Braccini T, Ricca E, et al. On the fate of ingested *Bacillus* spores. *Res Microbiol* 2000; 151: 361-68.
- [5] Nielsen P, Fritze D, Priest FG. Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. *Microbiology* 1995; 141: 1745-61.
- [6] Senesi S, Celandroni F, Tavanti A, Ghelardi E. Molecular characterization and identification of *Bacillus clausii* strains marketed for use in oral bacteriotherapy. *Appl Environ Microbiol* 2001; 67: 834-39.
- [7] Mazza G. Genetic studies on the transfer of antibiotic resistance genes in *Bacillus subtilis* strains. *Chemioterapia* 1983; 2: 64-72.
- [8] Mazza P, Zani F, Martelli P. Studies on the antibiotic resistance of *Bacillus subtilis* strains used in oral bacteriotherapy. *Boll Chim Farm* 1992; 131: 401-408.
- [9] Duc LH, Hong HA, Barbosa TM, Henriques AO, Cutting SM. Characterization of *Bacillus* probiotics available for human use. *Appl Environ Microbiol* 2004; 70: 2161-71.
- [10] Urdaci MC, Bressollier P, Pinchuk I. *Bacillus clausii* probiotic strains: antimicrobial and immunomodulatory activities. *J Clin Gastroenterol* 2004; 38: 86-90.
- [11] Cenci G, Trotta F, Caldini G. Tolerance to challenges miming gastrointestinal transit by spores and vegetative cells of *Bacillus clausii*. *J Appl Microbiol* 2006; 101: 1208-15.
- [12] Fakhry S, Sorrentini I, Ricca E, De Felice M, Baccigalupi L. Characterization of spore forming *Bacilli* isolated from the human gastrointestinal tract. *J Appl Microbiol* 2008; 105: 2178-86.
- [13] Ciffo F. Determination of the spectrum of antibiotic resistance of the "*Bacillus subtilis*" strains of *Enterogermina*. *Chemioterapia* 1984; 3: 45-52.
- [14] Abbrescia A, Martino PL, Panelli D, et al. The respiratory chains of four strains of the alkaliphilic *Bacillus clausii*. *FEBS Open Bio* 2014; 4: 714-721.
- [15] Lippolis R, Gnoni A, Abbrescia A, et al. Comparative proteomic analysis of four *Bacillus clausii* strains: Proteomic expression signature distinguishes protein profile of the strains. *J Proteomics* 2011; 74: 2846-55.
- [16] Lippolis R, Siciliano RA, Mazzeo MF, et al. Comparative secretome analysis of four isogenic *Bacillus clausii* probiotic strains. *Proteome Sci* 2013; 11: 28.
- [17] Gueimonde M, Sánchez B, de Los Reyes-Gavilán CG, Margolles A. Antibiotic resistance in probiotic bacteria. *Front Microbiol* 2013; 4: 202.
- [18] European Food Safety Authority (EFSA). Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. *The EFSA Journal* 2008; 732: 1-15.
- [19] Courvalin P. Antibiotic resistance: the pros and cons of probiotics. *Dig Liver Dis* 2006; 38: 261-265.
- [20] Bozdogan B, Galopin S, Leclercq R. Characterization of a new *erm*-related macrolide resistance gene present in probiotic strains of *Bacillus clausii*. *Appl Environ Microbiol* 2004; 70: 280-84.
- [21] Bozdogan B, Galopin S, Gerbaud G, Courvalin P, Leclercq R. Chromosomal *aadD2* encodes an aminoglycoside nucleotidyl transferase in *Bacillus clausii*. *Antimicrob Agents Chemother* 2003; 47: 1343-46.
- [22] Galopin S, Cattoir V, Leclercq R. A chromosomal chloramphenicol acetyltransferase determinant from a probiotic strain of *Bacillus clausii*. *FEMS Microbiol Lett* 2009; 296: 85-89.
- [23] Nair J, Rouse DA, Bai GH, Morris SL. The *rpsL* gene and streptomycin resistance in single and multiple drug-resistant strains of *Mycobacterium tuberculosis*. *Mol Microbiol* 1993; 10: 521-27.
- [24] Vogler AJ, Busch JD, Percy-Fine S, Tipton-Hunton C, Smith KL, Keim P. Molecular analysis of rifampin resistance in *Bacillus anthracis* and *Bacillus cereus*. *Antimicrob Agents Chemother* 2002; 46: 511-13.
- [25] Young VB. The intestinal microbiota in health and disease. *Curr Opin Gastroenterol* 2012; 28(1): 63-9.
- [26] Candela M, Consolandi C, Severgnini M, et al. High taxonomic level fingerprint of the human intestinal microbiota by ligase detection reaction universal array approach. *BMC Microbiology* 2010; 10: 116.
- [27] Hoyles L, Honda H, Logan NA, Halket G, La Ragione RM, McCartney AL. Recognition of greater diversity of *Bacillus* species and related bacteria in human faeces. *Res Microbiol* 2012; 163: 3-13.
- [28] Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010; 464(7285): 59-65.
- [29] Magrone T, Jirillo E. The interplay between the gut immune system and microbiota in health and disease: nutraceutical intervention for restoring intestinal homeostasis. *Curr Pharm Des* 2013; 19(7): 1329-42.
- [30] Girlich D, Leclercq R, Naas T, Nordmann P. Molecular and Biochemical Characterization of the Chromosome-Encoded Class A beta-Lactamase BCL-1 from *Bacillus clausii*. *Antimicrob Agents Chemother* 2007; 51: 4009-4014.
- [31] Roberts MC. Update on acquired tetracycline resistance genes. *FEMS Microbiol Rev* 2005; 245: 195-203.
- [32] Drago L, Mattina R, De Vecchi E, Toscano M. Phenotypic and genotypic antibiotic resistance in some probiotics proposed for medical use. *Int J Antimicrob Agents* 2013; 41: 396-97.