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Full Length Research Paper

Bifidobacterium spp.: A unique etiopathogenic agent for intra-abdominal infections

A. M. Israil[1], R. S. Palade[2*], M. C. Chifiriuc[1], C. Delcaru[1], D. Voiculescu[2], D. Popa[2], and D. Davitoiu[2]

[1]Cantacuzino Institute, Bucharest, Romania.
[2]First Surgery Clinic of the University Hospital, Bucharest, Romania.

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***Bifidobacterium* spp. are non-sporulating Gram-positive anaerobic bacilli that can grow also in microaerophilic conditions and are constituting part of normal enteral flora, playing a complex role in the maintenance of the health state of the human organism. However, in recent times, the literature has reported that in certain conditions (advanced age, immunodeficiency status, co-morbidities etc), commensal *Bifidobacterium* strains can turn into pathogens, being implicated in different infectious processes (pancreatitis, peritonitis, neonatal meningitis, aspiration pneumonia), sometimes with severe, life-threatening evolution. In the present study, there are reported five severe clinical cases of abdominal surgical emergencies, in which the *Bifidobacterium* strains were isolated as the unique etiological infectious agents, exhibiting at the same time, a large profile of virulence factors and high resistance to second generation cephalosporins, meropenem and ertapenem. All five strains exhibited susceptibility to imipenem, amoxicillin plus clavulanic acid, piperacillin plus tazobactam and ticarcillin plus clavulanic acid.**

Key words: Intra-abdominal infections, *Bifidobacterium* spp., virulence, antibiotic resistance.

INTRODUCTION

Bifidobacterium spp. non-sporulating, Gram-positive anaerobic bacilli that can grow also in microaerophilic conditions are constituting part of normal human microbiota of the mouth (Crociani et al., 1996), respi-ratory and enteral tract (Bailey and Scott, 2002). Usually considered as commensal enteral bacteria with low virulence, with probiotic and anticarcinogenic properties, *Bifidobacterium* sp. is responsible for deconjugation /hydrolysis process of conjugated bile acids (Picard et al., 2005). During the last two decades, the literature has cited more and more frequently, clinical cases in which the *Bifidobacterium* spp. strains have been isolated from anaerobic pancreatic infectious processes (2.5% of cases) (Brook and Frasier, 1993; Verma et al., 2010), pseudocysts (as complications in 10% cases of pancreatitis), peritonitis, aspiration pneumonia (Green, 1978), neonatal meningitis (Nakazawa et al., 1996) and septicemia (Cryan et al., 1991; Dorsher et al., 1991; Gyoungym et al., 1999).

The purpose of this study was to identify and to demonstrate the implication of *Bifidobacterium* strains in five severe clinical cases of infections associated with surgical abdominal emergencies. These five cases were selected from a total number of 119 randomized cases, based upon the isolation of the respective bacteria as the unique etiological agent as well as on the increased expression of virulence factors and antibiotic resistance markers of these strains.

MATERIALS AND METHODS

Specimens and culture media

Clinical specimens represented by peritoneal cavity liquid were collected during the surgical procedures from 119 cases of abdominal surgical emergencies. Transport/preservation media: Carry Blair and nutritive broth were used for the transport of aerobic, while thioglycholate broth and Schaedler medium for

*Corresponding author. E-mail: carmen_balotescu@yahoo.com.

anaerobic strains.

Culture media

5% sheep blood agar and MacConkey agar were used for the cultivation of aerobic and Brucella blood agar (supplemented by hemin 5 µg/ml and vitamin K 1 µg/ml) and bile esculine agar for anaerobic bacteria. Anaerobic conditions were provided by incubating the culture media in anaerobic bags or in an exicator containing catalyst substance.

Special media for the assessment of different virulence factors: 5% sheep blood agar for haemolysins, 15% casein agar for caseinase, 3.5% gelatin agar for gelatinase, porcine gastric mucine in brain heart agar with 2% NaCl for mucinase, DNA-agar for DN-ase, 10% starch agar for amylase, 1% Tween-80 agar for lipase, 2.5% yolk agar for lecithinase, Wagatsuma agar for Kanagawa haemolysin,1% aesculin iron salts for esculethol (an iron-chelating agent), nutrient broth to assess the ability of the microbial strains adherence to abiotic surfaces (slime test) (Israil et al., 2003; Israil et al., 2010).

Brucella blood agar (supplemented by hemin 5 µg/ml and vitamin K 1 µg/ml) was used for testing the antibiotic susceptibility.

The clinical specimens were collected intraoperatory by syringue and inoculated into Cary Blair, Schaedler and thioglycolate media. Each set of samples was accompanied by the corresponding clinical files providing certain data about the patient (identification code, age, sex, diagnostic, preoperatory empiric antibiotic treatment, immunologic status, co-morbidities, type of sample).

These data allowed us to classify the studied cases as related to their severity level (Carmeli score) (Slavcovici et al., 2009) in three risk groups, as follows: *risk group I*: young persons, without previous contact with the hospital environment, without treatment, without co-morbidities, with community – acquired infections; *risk group II*: aged persons (> 65 years old) with previous contact with the sanitary system, recent antibiotic therapy, with co-morbidities, with microbial infections produced by bacterial strains with antibiotic resistance and *risk group III*: persons with previous long hospitalization, invasive procedures associated with acute severe immunodeficiency syndrome, neoplasm cases, chronic renal failure, *diabetes mellitus*, nosocomial infections with hospital multidrµg resistant strains.

At the laboratory, the samples collected in Cary Blair medium were streaked for isolation on 5% sheep blood agar and Mac Conkey agar and incubated aerobically at 37ºC, for 48 to 72 h, whereas those collected in Schaedler and thioglycolate media were streaked on Brucella blood agar (supplemented as already men-tioned) and on selective bile esculine agar, incubated in anaerobic bags with catalyst substance kept in exicator at 35 to 37ºC until four weeks, with daily examination for the bacterial growth (Bailey and Scott, 2002).

Bacteriological identification

Macroscopic examination of the isolated colonies on Brucella blood agar was performed and the characteristic *Bifidobacterium* colonies (small, white, convex, shiny, with irregular edge) were selected for microscopic and biochemical identification. On bile esculine agar, the difference between the esculine positive (black) and esculine negative colonies was done.

Microscopic examination of Gram stained smears evidenced: Gram positive diphteroid, coccoid or thin, pointed shape or larger, highly irregular, curved rods with branching, rods terminated in clubs or thick, bifurcated (forked) ends (“dog bones”) (Bailey and Scott, 2002).

Biochemical identification of the colonies was performed by API 20 A galleries and other tests motility (negative), growth on bile medium (negative), nitrate reduction (negative). The reading and interpretation of the results were done after 48 h -7 days incubation at 37°C in anaerobic conditions.

In the cases in which on the first plating from the transport/ preservation media, it was not possible to isolate any bacteria, a new inoculation from the same preservation anaerobic media into a new regenerated thioglycolate broth was done and after anaerobic incubation at 35 to 37°C for 48 h to 7 days, the grow n culture was streaked on Brucella agar just in the same conditions as mentioned earlier.

The assessment of 12 virulence factors was done using the methods already mentioned (Israil et al., 2003; 2010).

Antibiotic susceptibility testing of the isolated strains was done on Brucella blood agar (supplemented with 5 µg/ml hemin and 1µg/ml vitamine K) by disk diffusion method adapted to anaerobic conditions, that is, a portion of one colony of the tested anaerobic strain was transferred to Brucella blood agar plate. The Petri plate was streaked several times with the respective strain to produce a heavy lawn of bacterial growth. Thereafter the antibiotic disks were placed at a distance of 30 mm on the surface of the respective Petri plates. A quadrant of a plate was streaked only with the tested strain (without any antibiotic disk being added) for being used as control of bacterial growth. All plates were incubated anaerobically for 48 hrs at 35 ^{o}C (Bailey and Scott, 2002).

The antibiotics were chosen in accordance with CLSI recommendations (CLSI, 2007): penicillin P (10 I.U./disk), piperacillin PIP (100 µg), amoxicillin plus clavulanic acid AUG (20µg/10 µg), ampicillin plus sulbactam SAM (10µg/10µg), cefoxitin FOX (30 µg), clindamycin CC (2 µg), ertapenem ETA (10 µg), meropenem MER (10 µg), imipeneme IMP (10 µg), linezolid LZ (30 µg), piperacillin plus tazobactam TZP (100µg/10 µg), ticarcillin plus clavulanic acid TIM (75 µg/10 µg), to which there were added: cefotaxime CTX (30 µg), erithromycin E (15 µg) and gentamycin GM (10 µg).

Reading of the results: only if the control cultures were grown, the inhibition areas of the bacterial growth around the disks were taken into consideration, that is an inhibition area of 10 mm or less was considered as resistance whereas an inhibition area greater than 10 mm as susceptibility aspect (Bailey and Scott, 2002; CLSI, 2007).

RESULTS AND DISCUSSION

The clinical aspects of the five selected cases, their classification in the three risk groups, as well as the applied surgery procedures and treatment are presented in Table 1.

Out of the 119 randomized studied clinical cases of abdominal surgical emergencies, *Bifidobacteium* strains were isolated as unique bacteria in five (4.13%) cases. In normal conditions, the stomach cavity is considered almost a sterile compartment, the ingested penetrating microorganisms being destroyed by the gastric acidity. In the bowel, the bacteria multiplication is inhibited by certain factors, such as: enteral peristaltic waves as well as substances inhibiting the bacterial growth.

In case of intestinal tract obstruction, the ileon microbiota becomes similar with that of the colon *(Bifidobacterium* spp, *Clostridium* sp., *E. coli, Enterococcus* sp.); the human colon being populated by a very large spectrum of microbial ecosystem (up to 10^{13} to 10^{14} CFU/g) constituted of several hundred species (Guarner and Malagelada, 2003).

It must be taken into account that as ubiquitous members of human normal enteral flora, anaerobic organisms may contaminate clinical materials and for this reason, to assign the clinical significance of anaerobic bacteria isolated in laboratory is very important and often very difficult.

The *Bifidobacterium* spp is not commonly found in clinical specimens, but only usually encountered with mixed infections of abdomen, pelvis or genitourinary tract (Bailey and Scott, 2002). The virulence factors of this anaerobic species are until at present not completely known.

Many anaerobic infections involve mixture of anaerobic and facultatively anerobic organisms (as Enterobacteriaceae spp), so that it becomes very difficult to appreciate the extent to which a particular anaerobic species is contributing to infection. In our laboratory the *Bifidobacterium* strains were isolated, in five cases, as the unique bacterial etiology. The exclusive presence of *Bifidobacterium* spp. in the absence of all other anaerobic and facultatively anaerobic (Enterobacteriaceae) micro-biota is pleading for an increased pathogenic potential of these strains, due to a high multiplication rate inhibiting all other anaerobic and aerobic species and to the expression of different virulence factors.

In all five cases the *Bifidobacterium* strains exhibited the presence of at least 1 to 8 virulence factors (Figure 1) aspect pleading for the emergence of virulence ability in these bacteria, demonstrating that the commensal *Bifidobacterium* spp. could turn into pathogenic, generating the respective infectious processes. Mucinase was present in all five isolated strains, followed by esculethol (an iron chlating agent) production in 3 strains and lecithinase, caseinase, gelatinase and haemolysins in two strains. To this process of turning from non-pathogenic into pathogenic strains could also have contributed some host factors, such as: the advanced age of the patients (by an involution process of the organism normal reactivity) and the presence of different co-morbidities (pulmonary fibrosis, pachypleuritis, cardio-megaly, renal failure, neoplasic processes, metabolic diseases as diabetes mellitus and obesity).

Preoperatory empiric antimicrobial therapy was instituted in three of the five cases; this treatment was applied simultaneously with the surgical procedure. Four patients were surgically recovered in 10 to 29 days, one patient (case no. 3) being transferred at request one day after the surgical intervention could not be postoperatory followed up.

The results of the antibiotic susceptibility testing of the five *Bifidobacterium* spp strains indicated (Table 2) that all five strains exhibited resistance to cefoxitin, ertapenem, meropenem, three strains to penicillin, and only one to each of the following antibiotics: amoxicillin, ampicillin+sulbactam, piperacillin, cefotaxim and clindamycin.

All strains remained sensitive to imipenem, augmentin (ampicillin plus clavulanic acid), erythromycin, gentamycin, linezolid, tazobactam plus piperacillin, ticarcillin plus clavulanic acid. Concerning the three clinical cases submitted to preoperatory treatment, the results of antibiotic susceptibility testings indicated that the strains were susceptible to the selected antibiotics (Table 1).

The present cases demonstrated that the commensal enteral bacteria like *Bifidobacterium spp*., usually as part of colonic microbiota (Gibson and Roberfroid, 1995) in certain anormal conditions (i.e. advanced age, co-morbidities, metabolic diseases, depressed immunitary system) may turn into pathogenic. In intraabdominal infections, especially when the integrity of the enteral wall is compromised by the pathological process, occurs a massive contamination (see case no. 3) of the normal sterile areas of the organism, with aerobic/anaerobic colic flora, including here also *Bifidobacterium* strains.

It must be pointed out that the primary infection generated by aerobic/ anaerobic bacteria is a dynamic process, its evolution being dependent upon multiple factors resulting in the selection of certain bacterial strains. The emergence of resistance, virulence, multipli-cation ability, toxinogenesis, invasivity are determinant factors for the aggressive behavior of the respective selected bacteria at a certain favorable moment.

The highest number of anaerobic abdominal infections occurs when a patient's normal endogenous flora gains access to a sterile site of the body as result of disruption of certain anatomic barriers (surgery procedures, accidental trauma etc) or alteration of host defense mechanisms (diabetes, malignancies, immunosuppressive therapy, aspiration).

In the last years, since the prophylactic probiotics and probiotic supplemented food having been widely used, a shift from Gram-negative to Gram-positive anaerobic bacteria has been probably occurred, the enteral Gram-negative flora (*Bacteroides* sp.) being substituted by Gram-positive, non-sporulating bacteria (*Bifidobacterium* sp.), these aspect probably favoring the more frequent implications of *Bifidobacterium* sp. strains in intra-abdominal infectious processes in special clinical conditions of the host organism.

As commensal bacteria, in normal conditions *Bifidobacterium* spp. are provided with ability of adherence to the enterocyte surfaces, playing important roles as probiotics, in association with other enteral species (lactobacilli, enterococci*, Sacharomyces cerevisiae*), by blocking the adherence of pathogenic agents to the enteral wall and thus preventing the infectious processes (Lievin et al., 2000; FAO/WHO, 2001; Borriello et al., 2003), stimulating the immune res-ponse (Gibson and Wang, 1994; Gibson, 2003) and by preventing the colon carcinogenesis process by inhibiting the synthesis of enzymes (beta-glucuronidase, azoreductase, nitroreductase) responsible for turning out the pro-carcinogenic into active carcinogenic factors.

Table 1. Description of the five clinical cases in which *Bifidobacterium* strains were isolated as unique etiology.

File case no.	1	2	3	4	5
Age	**62**	**69**	**32**	**56**	**84**
Sex	**Male**	**Male**	**Male**	**Female**	**Female**
Complaints	Diffuse bdominal pains. Vomiting Nausea	Right hypochondrium pains Nausea Bilious-alimentary vomiting Fever Chills	Diffuse abdominal pains Nausea Vomiting lockage of the intestinal transit for two ays	Diffuse abdominal pains two days before hospitalization Nausea Vomiting	Diffuse abdominal pains Nausea Impaired general conditions Fever
Past medical history		Apendicectomy	Congenital hypoacusis		Left breast operated cancer Total histerectomy
History of drinking alcohol/smoking	Smoking	Absent	Absent	Alcoholism Smoking	Absent
Physical exam	Diffuse abdominal pains. Abdominal muscular defence.	Pains in the epigastric area and in the right hypochondrium with muscular defense	Abdominal meteorism Peritoneal irritation Normal blood pressure Polypneea Oliguria	Generalized abdominal muscular defense General condition impairment Irritability Non-cooperant patient "live pains"	Abdominal meteorism Diffuse abdominal pains to palpation Muscular defense in hypogastrum and right iliac flank
Clinical diagnosis	Perforated ulcer of the anterior duodenum bulb Old non-treated diffuse acute peritonitis	Gangrenous lithiasic acute cholecystitis Shirt-front pericholecystitis	Sigmoidian volvulus with sigmoid loop complete necrosis Secondary intestinal occlusion Acute diffuse peritonitis	Perforated callous ulcer at the small gastric curvature Old secondary diffuse peritonitis	Purulent acute diffuse peritonitis Pelvi-under-umbilical plastic peritonitis Perforated and over-infected large-size rectal malignant tumor
Co-morbidities	Cardiomegalia, Pulmonary fibrosis Left antero-basal pachypleuritis Left inguinal hernia Acute conjunctivitis	Ischaemic coronary disease Cholecyst chronic lithiasis Chronic atrial fibrillation Non-treated high blood pressure II-nd level Chronic sthmatic bronchitis	Secondary acute renal failure	Chronic obstructive broncho-pneumonia	Gall –bladder lithiasis High blood pressure II- nd level Ischaemic coronary disease
Immunodepression	*De novo diabetes mellitus* second type Obesity of second type	*De novo diabetes mellitus* second type Obesity of second type Septic status	Multiple organ failure Septic status	*De novo diabetes mellitus* of second type Septic status	Severe sepsis status

However, in abnormal conditions, *Bifidobacterium* spp. may ascend from the colon into the upper part of the enteral lumen, penetrate through the enteral wall into the peritoneal cavity, multiply and become responsible for severe clinical cases, as demonstrated also by our results. In 2010, Schneider et al., 2010 mentioned that

Table 1. Contd.

Risk group	**II**	**II**	**III**	**II**	**III**
Paraclinic exams: Imagistic diagnosis Laboratory exams	Echography: non-lithiasic colecyst. Liquid among the intestinal loops and in Douglas space WBC:5580 /mm3 Hb.:17,9 g Hematocrit=53,5 % PLT=260.000/mm3 Glycemia: 167,55mg/dL Urea =44,5 mg/dL	Inspissated cholecyst wall (8 mm) Parietal oedema Multiple hyperecogenic images WBC: 20.100 /mm^3 Hb.:16 g % Hematocrit:47,6% PLT =249.000/ mm^3 Na:137/mmol L K:4.4/mmol L Glycemia=342.2 mg/dL Urea =62,7 mg/dL Creatinine-106mg/dL EKG=chronic atrial fibrillation, diffuse ischemic alterations	Empty rectal ampoule Distended loop Occlusion WBC: 5470/mm^3 Hb: 15.2 g/% PLT: 252.000/ mm^3 Glycemia: 91 mg% Urea; 121 mg% Creatinine: 2.05 mg% Na 134 mmol/L K: 3.9 mmol/L	Rx : normal thoracoabdominal aspect Ecography; normal aspect Secondary leucopenia WBC: 1940 - 2200/mm^3 Hb: 13.4g% Hematocrit: 39.6g% PLT: 275.000/ mm^3 Na: 134 mmol/L K: 4.6 mmol/l Glycemia: 149.2 mg% Urea: 42.1 mg% Creatinine: 1.26 mg%	Distended ileal loop- ileus Average/high amount of peritoneal liquid Bilateral pneumoperitoneum WBC: 26.300 mm Hb: 10.5g% Hematocrit: 32.5g% PLT: 489.000/ mm Na: 130 mmol/L K: 3.56 mmol/l Glycemia: 73 mg% Urea: 62 mg% Creatinine: 0.8 mg%
Preoperatory empiric treatment	Absent	Hydroelectrolytic substitution therapy with Ringer solution cefoperazone+sulbactam 2v/day × 1 day Gentamycin (GM) 2 v of 80mg/day × 1 day	Hydroelectrolytic substitution therapy with sterile saline SAM 3 v/ day x1day	Hydroelectrolytic substitution therapy with Ringer solution SAM 3 v/day	Hydroelectrolytic substitution therapy with Ringer solution SAM 2 v/day 1 day and ceftriaxone 1 g/day x 2 days
Surgical procedure	Exploratory laparatomy Suture of the the ulcerous perforation with epiploono-plasty Toilet and multiple peritoneal drainages	Viscerolysis Anterograde cholecystectomy Subhepatic toilet and drainages	Sigmoidecto-my, Hartmann method Peritoneal toilet and drainages	Suture of the ulcerous perforation Gastric biopsy Peritoneal toilet and drainage	Exploratory laparatomy Viscerolysis Maydl procedure (left iliac anus by glass rod) Multiple peritoneal toilet and drainages
Intraoperatory collected specimens	Peritoneal purulent content	Peritoneal purulent content	Peritoneal purulent content	peritoneal content	Peritoneal purulent content
Bacteriological identification	*Bifidobacterium* spp.	*Bifidobacterium* sp.	*Bifidobacterium p.*	*Bifidobacterium* sp.	*Bifidobacterium* sp.
Antibiotic susceptibility to :	*AUG, E, GM, IMP, LZ, AMX, SAM, CTX, CC, PIP, TZP, TIM*	*AUG, E, GM, IMP, LZ, AMX, SAM, CTX, CC, PIP, TZP, TIM*	*AUG, E, GM, IMP, LZ, P, CC, PIP, TZP, TIM*	*AUG, E, GM, IMP, LZ, P, AMX, SAM, CTX, CC, PIP, TZP, TIM*	*AUG, E, GM, IMP, LZ, AMX, SAM, CTX, CC, PIP, TZP, TIM*

Table 1. Contd.

Postoperatory treatment	cefoperazone+sulbactam 2 v/ day for 5 days followed by Cefuroxime 3 v/day for 6 days	TZP 3v/day for 6 days	ETM 1g/day for 6 days Metronidazole 500 mg/8 h for 1 day	SAM 3 v/day for 8 days	SAM 3v/day for 7 days followed by TZP 3 v/day for 3 days
Postoperatory evolution	Fever 38-38.5 ^{0} C after 3-4 days Good evolution after antibiotic treatment	Uneventful course	The patient could not be followed up, leaving the hospital at request one day after having been operated.	eventful course	Uneventful course
Hospitalization period (duration)	11 days	12 days	1 day	10 days	29 days
Patient clinical state when leaving the hospital	Recovered	Recovered		Recovered	Recovered
Special considerations	Post-operatory antibiotic treatment with cefoperazone+sulbactacm followed by cefuroxime		Transferred at request one day after surgery in a county hospital		Postoperatory antibiotic treatment with SAM followed by TZP

the prophylactic use of probiotics in severe forms of intra-abdominal infections showed not only no improvement of the disease, but even an increased mortality," the reason of mortality remaining still unclear". In the light of our results, the pathogenic potential of *Bifidobacterium* sp. strains in certain host conditions, may trigger a severe outcome of the respective clinical cases.

Conclusion

The present study demonstrated that a commensal anaerobic species like *Bifidobacterium* strains, which in normal conditions is playing an important role in the maintenance of the health condition of the human host, in certain abnormal cases can turn into pathogenic, becoming responsible for life-threatening infections.

The presence of the virulence factors in the *Bifidobacterium* strains isolated as unique etiology and the precarious reactivity of the patients favored the switch from commensal to pathogenic. All the five acute peritonitis were old non-treated cases, four of them displaying also a diffuse form and one being complicated with shirt-front cholecystitis and different immuno-depression conditions (septic status, diabetes mellitus, obesity, multiple organ failure). The pathogenic *Bifidobacterium* strains implicated in the five clinical cases of surgical abdominal emergencies proved to be all resistant to penicllins, second generation cephalosporins, ertapenem and meropenem, ampicillin plus sulbactam, and were susceptible to imipenem, amoxicillin plus clavulanic acid, gentamycin, piperacillin plus tazobactam and ticarcillin plus clavulanic acid.

The antibiotics used for the preoperatory therapy corresponded perfectly to the antibiotics that have been indicated as active by the results of the antibiotic susceptibility testing of the respective isolated bacterial strains.

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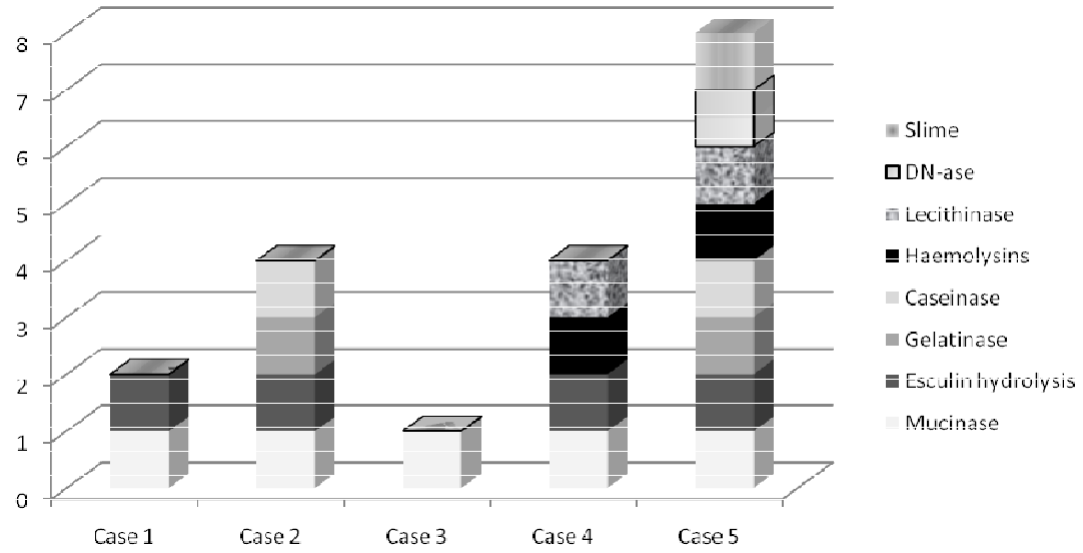

Figure 1. The patterns of virulence factors in the five *Bifidobacterium* strains.

Table 2. Distribution of the resistance patterns (grey cells) among the five *Bifidobacterium* spp. strains.

Antibiotic	Strain no. 1	Strain no. 2	Strain no. 3	Strain no. 4	Strain no. 5
Cefoxitin					
Ertapenem					
Meropenem					
Penicillin					
Amoxicillin					
Ampicillin+sulbactam					
Piperacillin					
Cefotaxim					
Amoxicillin+clavulanic acid					
Ticarcillin+tazobactam					
Ticarcillin+clavulanic acid					
Imipenem					
Clindamycin					
Erithromycin					
Gentamycin					
Linezolid					

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