Sigma J Eng Nat Sci, Vol. 43, No. 3, pp. 1014–1019, June, 2025

Sigma Journal of Engineering and Natural Sciences
Web page info: https://sigma.yildiz.edu.tr
DOI: 10.14744/sigma.2025.00088

Research Article

Study of the antibiotic resistance of some bacterial strains isolated from the gut microbiota of children fed with commercial infant formula

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ARTICLE INFO

Article history
Received: 14 December 2023
Revised: 15 March 2024
Accepted: 10 June 2024

Keywords:
Antibiotic Resistance, Children, Commercial Infant Formula; Gut Microbiota

ABSTRACT

The intestinal microbiota is a set of microorganisms in equilibrium, which naturally shelter the digestive tract, and fulfill several functions beneficial to the host. However, this equilibrium can be influenced by many factors such as the type of diet.For instance, the choice of feeding, whether it's breast feeding or the use of commercial infant formula, profoundly influences the selection and establishment of microbial strains in the infant's digestive system. In particular, this microbial strain selection can also contribute to the emergence of antibiotic resistance, posing a significant public health issue and consequently limiting therapeutic options.
In this work, we considered it essential to examine the profile of the antibiotic resistance of five (05) bacterial strains isolated from the stools of children fed with commercial milk aged 2 years (02) and residing in the Mascara province (Algeria), against sexes (6) antibiotics commonly used in pediatrics. The results obtained give us evidence that the isolated strains show a marked resistance towards almost all antibiotics. In addition, we noted that 88,88% of isolated strains are hyper-productive of extended-spectrum β-lactamase. Whereas, cephalosporinase production was detected only in (*E.faecium*) and that the combination erythromycin and gentamicin revealed a phenotype of antagonism.

Cite this article as: Asmaa B, Amine AM, Mirvette B, Aicha TT. Study of the antibiotic resistance of some bacterial strains isolated from the gut microbiota of children fed with commercial infant formula. Sigma J Eng Nat Sci 2025;43(3):1014–1019.

INTRODUCTION

The digestive tract is an extremely complex ecosystem where the three main players - microflora, nutrients and host cells are in permanent contact and interact. These micro-host-host relationships have long been viewed primarily from the perspective of pathogenicity.

However, more and more studies are showing beneficial interactions between commensal flora and the human body, making flora a true partner [1].

This highly complex microflora is therefore an active element of intestinal physiology and the importance of this flora has led for several years to its characterization, in order to find relevant answers on the rise of some pathology

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This paper was recommended for publication in revised form by Editor-in-Chief Ahmet Selim Dalkilic

Published by Yıldız Technical University Press, İstanbul, Turkey

[2,3] and to better control the factors that influence; ensuring a certain level of safety for at-risk populations such as new-borns.

Certainly, one of the factors that has a noteworthy impact on both the quality and quantity of the intestinal flora is the kind of milk given to children [4].

Indeed ; Breast milk is renowned for its abundance of essential nutrients, including specific oligosaccharides that promote the growth of beneficial bacteria in the digestive tract of infants [5].

This natural diet plays a pivotal role in the development of a healthy and balanced intestinal flora. On the other hand, the use of commercial formula, although it may be a necessary alternative in certain situations, does not always provide the same beneficial components and oligosaccharides as breast milk. This can have consequences for the diversity and stability of the intestinal flora, which may potentially influence the long-term health of newborns, including their susceptibility to infections and gastrointestinal issues [6-8].

However; the rise of antibiotic resistance in neonates has become a growing concern in the field of healthcare. Neonates are particularly vulnerable to infections, and antibiotics are often a critical component of their medical care.

Nonetheless, the indiscriminate use of antibiotics, both in neonatal care and in the broader healthcare land scape, has contributed to the emergence of antibiotic-resistant strains of bacteria in this population. Which can poses a significant challenge for neonatal healthcare providers.

To address this issue, curents studies are crucial to understand the factor contributing to antibiotic resistance in neonates, as well as finding strategies to mitigate its impact.

MATERIALS AND METHODS

Collection of Samples

The study focused mainly on stool samples from children fed with commercial infant formula; aged 2 years (02) and residing in the Mascara province (Algeria).

The stool samples of the children were collected according to the repository in Medical Microbiology [8] in sterile sealed and labeled boxes. The transmission was immediately made to the analysis laboratory via coolers (+ 4°C).

Moreover; and for scientific rigor, our study was conducted according to the modalities and methodologies of clinical trials. An informed consent of the parents were conceived by respecting the Belgian law of September 8, 1992 relating to the protection of the private life with regard to the processing of personal data [9].

Inclusion and exclusion criteria were used in order to standardize any exogenous factors that may influence the interpretation of our results, we have included in this analysis:

- Children of both sexes, aged two (02) years, residing in the province of Mascara; and fed with the same brand of commercial infant formula.

However; we have excluded from this study:

- children outside the age groups, fed with breast milk; or those who consumed antibiotics during the sampling period

METHODOLOGY

Isolation of Strains

Strains were characterized by stool culture according to the following steps:

- Selective isolation of selected strains from decimal dilutions of stool, according to the usual techniques of medical bacteriology [10; 11].

The general procedures adapted in our study are:

A/ Preparation of the stock solution and decimal dilutions: Prepare various dilutions of the stool sample by combining measured amounts of the sample with a specific volume of diluting agent (typically sterile saline). These dilutions should range from 10^{-1} to 10^{-6}.

b/ Inoculation onto selective medium : each previous dilution was inoculated onto an appropriate selective mediium. Here are the main selective media used in our study.

- **Mac Conkey agar and Eosine methyl bleu agar** : Selective for Gram-negative bacteria
- **Bile Esculin Azide agar:** Selective for *Enterococcus* species.
- **Mannitol salt agar:** Selective for *Staphylococcus.*

The plates are incubated at the appropriate temperature and conditions suitable for the growth of the target strains (incubation time typically ranges from 24 to 48 hours).

- Microscopic identification (Gram stain), physiological and biochemical identification of isolated strains was performed by the conventional microbiological identification galleries of each species(Api galleries) [11]

Study of the Antimicrobial Resistance of Identified Strains

The purpose of this step is to help in the therapeutic decision-making, and the epidemiological surveillance of the bacterial resistance which will later guide the probabilistic antibiotherapy. We have tested the sensitivity of all the strains identified (05 strains) with respect to different antibiotics (ATB) by several antibiogram techniques according to the recommendations of the French Committee of the Antibiogram of the French Society of Microbiology [8].

The techniques illustrated in this study are:

- Agar diffusion technique (swab seeding).
- Detection of narrow-spectrum β-lactamase production (iodometric method).
- Synergy test (search for cephalosporinase).
- Determination of the minimum inhibitory concentration (MIC) by the chessboard method: Only one

combination was tested, vancomycin (ATB A) and gentamicin (ATB B). These molecules are part of the therapeutic arsenal. The reading and the expression of the results was carried out after 18 hours of incubation. The determination of the index of FIC (fractional inhibitory concentrations) is done according to the following formula:

$$FIC = FICA + FICB = \frac{MCI\ of\ A\ with\ B}{MCI\ of\ A\ alone} + \frac{MCI\ of\ B\ with\ A}{MCI\ of\ B\ alone}$$

With:

- FIC A: corresponds to the ratio of the MIC of A with B on the MIC of A alone,
- FIC B: corresponds to the ratio of the MIC of B with A on the MIC of B alone.

Because of this, if the FIC index is:

- Less than 0.75, the combination **is synergistic,**
- equivalent to 1, it is **additive**
- between 1 and 2, it is **indifferent**
- greater than 2, it is **antagonist** [8]

Furthermore; it is important to point out that the antibiotics tested (BIORAD Brand) in this study were selected using a questionnaire sent to paediatricians taking charge of the study population, in order to give our study a real dimension of the existence or not of the antimicrobial resistance of the strains responsible for gastroenteritis in this service (Table 1).

RESULTS AND DISCUSSION

Characterization of Microbial Strains Responsible for Gastro-Enteritis

A total of five (05) bacterial strains were isolated and identified. The bacteriological study completed to the microbial species have revealed the presence of: *Escherichia coli, Serratia marcescens, Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus sub.aureus*

Outcome of Antimicrobial Resistance Study of Identified Strains

Agar Diffusion Technique

The evaluation of antibiotic resistance susceptibility of isolated species is of big importance, since it guides the choice of treatments and reduces the selection pressure exerted by antibiotics. We conducted antibiograms on the five discovered species, comparing them with several antibiotic disks. The results obtained are illustrated in the figure below (Fig. 1); noting that the declaration that a strain is resistant or not to such an antibiotic has been referred to the manual of the French committee of antibiogram [8].

With regard to these results, it reveals that the resistant nature of the nine strains isolated was as follows:

- Two (02) strains (40%) are resistant to 90-100% of these antibiotics.

Table 1. Antibiotics used in susceptibility testing

Families		Abbreviation on the disks	disks load (µg)
β-lactams	Penicillin	Oxacilin (OX)	1
	Cephalosporins	Cefazolin (CZ)	30
	3rd Generation Cephalosporins	Cefixime (CFM)	10
	Penicillins with beta-lactamase inhibitor	Amoxicillin/ clavulanic acid (AMC)	20
Aminoglycosids		Gentamicin (CN)	10
Macrolides		Erythromycin (E)	15

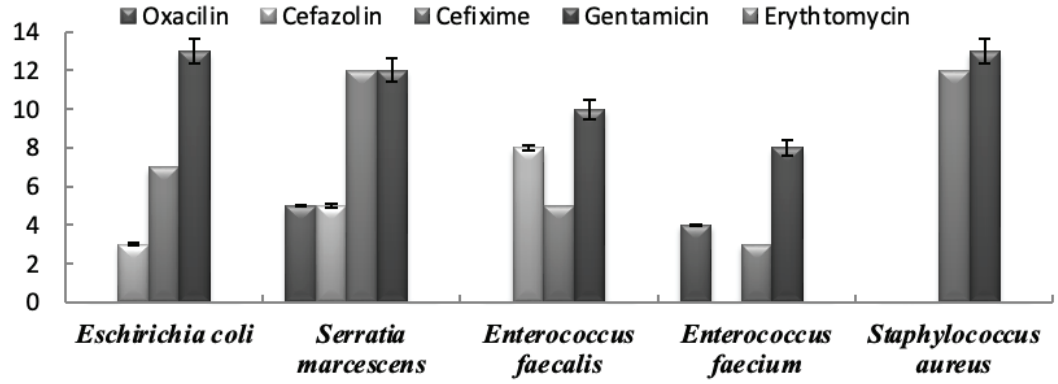

Figure 1. Result of antibiogram test.

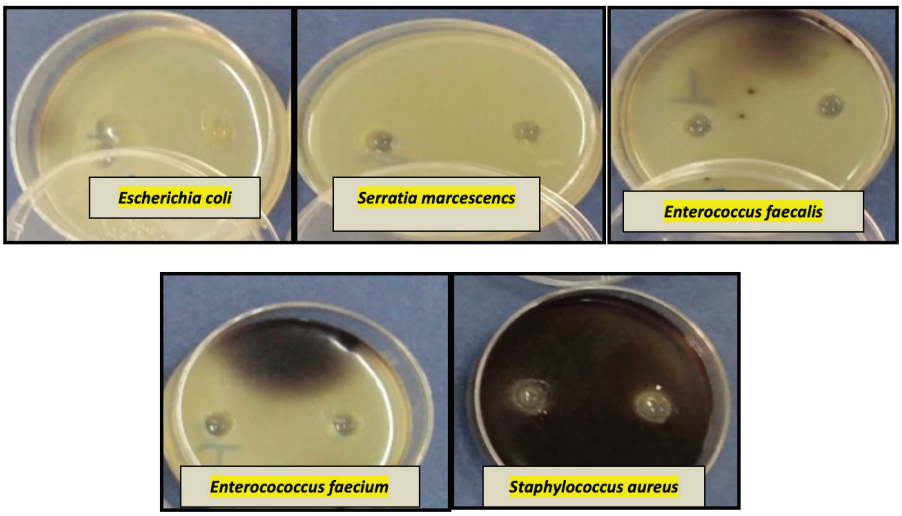

Figure 2. Results of narrow-spectrum β-lactamase detection.

✍ Three (03) strains (60%) represent a resistance rate varying between 50 and 80%.

Detection of Narrow-Spectrum Beta-Lactamase Production

The production of β-lactamase (penicillinase +) by the iodometric test was found infour microbial strains out of the five isolated strains with complete discoloration of the agar (Fig. 2), which could explain the resistance to certain β-lactams such as penicillin.

In practice, the detection of the different β-lactamases is based on the modification of the open β-lactam ring, accompanied by the formation of penicillic acid for penicillins, and cephalosporoid for cephalosporins, and the displacement of iodine. Iodide causes starch discoloration [12].

Although this iodometric test is a qualitative test, some strains are demarcated by a speed and a large production of β-lactamases.

Our result shows that only one strain; presented a negative penicillinase. So we can assume that these strains have other mechanisms of resistance not detectable by the iodometric test.

Synergy Test (Search for Cephalosporinase)

In our study; the presence of synergy was confirmed by the appearance of a zone of inhibition in the form of a characteristic champagne cork between clavulanic acid and cephalosporin of 3rd generation (cefixime) (Fig. 3).

This synergy test is based on the demonstration of a restoration of the activity of 3rd generation cephalosporins in the presence of an enzymatic inhibitor clavulanic acid.

Our results confirm the inefficacy of this association in 03 bacterial strains isolated from the children fed with commercial infant formula. This reinforces the postulate of multidrug resistance, whereas a positive synergy was clearly detectable in only 2 strains *(E. faecium* and *E. faecalis*).

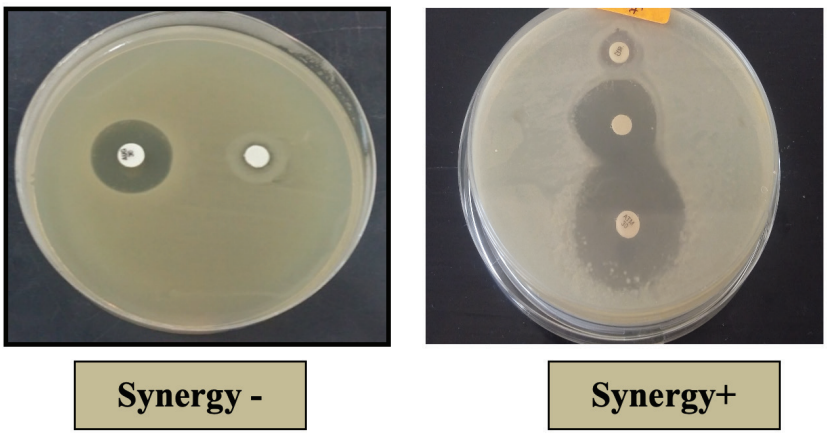

Figure 3. Results of synergy test.

Table 2. Results of the checkerboard analysis FIC index for the antibiotic combination

Strains	MIC of the ATB A alone (µg/ml)	MIC of the ATB A alone (µg/mL)	MIC of the ATB A with ATB B (µg/mL)	MIC of the ATB B with A (µg/mL)	FIC index	Signification
E.coli	0.125	4	0.125	16	5	Antagonist
Serratia marcescens	1	0.25	0.25	1	4.25	Antagonist
Staphylococcus aureus	0.25	32	2	16	8.5	Antagonist
E. faecalis	8	0.5	32	0.125	4.25	Antagonist
E. faecium	0.5	8	0.125	4	0.75	**Synergy**

ATB A: Gentamicin; ATB B: Erythromycin

The Chessboard Method

In order to increase bactericidal activity, to broaden the antibacterial spectrum, and even top revent the emergence of resistant mutants, the synergy between antibiotics is often required. For this, we have used the so-called "chessboard" method. The concentrations at which inhibition of bacterial growth occurs are noted and the interactions defined with calculations (Table 2).

Our results showed that the combination of gentamicin and erythromycin expressed antagonistic interaction in almost all strains tested. This illustrates an acquired multiresistance of the strains, which aggravates the clinical profile of these children. Noting that this combination has been very effective in dealing with only one strain (*E. faecium*).

Our study identified five (05) predominant bacterial strains in the stools of children who were fed commercial infant formula. The bacterial population isolated from the children participating in this study was primarily dominated by fecal enterococci (51.39% of the isolated population), predominantly *Enterococcus faecalis,* followed by Enterobacteria (32.91% of the isolated population). The qualitative and quantitative variations in the composition of these isolated strains may be attributed to the weakened barrier effect and reduced resistance to colonization in these children. This susceptibility makes the patients more vulnerable to infections, primarily caused by these two pathogens [13].

It is noteworthy that, despite *Enterococcus sp*. being a part of the normal flora of the gastrointestinal tract, as indicated by the work of [14], the observed variations underscore the dynamic nature of the microbial community in response to various factors such as the type of feeding.

From the results of the antibiogram shown above, we find a general multi resistance of the strains studied compared to antibiotics tested. On the other hand, this resistant is a cross-resistance to β-lactams which extends to other families of antibiotics thus reducing the choice of therapeutic molecules. All of the isolated organisms in this work were also resistant to Cefazolin and Oxaciline. These strains also escaped the action of, erthromycin reflecting resistance levels of 80%.

Moreover, antibiotic gentamicins were more potently active expressing sensitivity rates exceeding 55%. These results can be justified by the fact:

- An acquisition or overproduction of efflux pumps that can expel the antibiotic (often β-lactams and amino-glycosides) out of the cell itself against the concentration gradient.
- A modification of porins in Gram-negative bacteria, resulting in slower diffusion of β-lactams across the outer membrane [15].

However, the production of β-lactamase by the iodometric test was found in 80% of the strains tested. On the other hand, the resistance of the strains to the synergy test; may be justified by the presence of C3G-resistant bacteria suggestive of ESBL [16].

The search for synergy is usually justified only in situations where bactericidal activity is difficult to obtain with a single antibiotic as in our study.

For our strains, there is a demonstrated and constant antagonism between our two antibiotics (except for cases involving a strain with a low level of resistance) [17].This makes possible the following conclusion: our strains are multiresistant which implies a significant risk for the emergence of antimicrobial resistance and therapeutic failure.

CONCLUSION

Our study emphasizes the significant influence of feeding with commercial infant formula on the multi-drug resistance of gastro-intestinal strains to antibiotics in children. The results highlight the predominance of certain bacterial strains, particularly enterococci and enterobacteria, in children fed with this infant formula.

These observations suggest a possible relationship between dietary habits and the composition of the intestinal microbiota, with potential implications for antibiotic resistance. The weakening of the intestinal barrier and the reduction in resistance to colonization observed in these children could contribute to their increased vulnerability to bacterial infections, underscoring the importance of better understanding the impact of dietary choices on intestinal health and antibiotic resistance.

As we move forward, it's vital that we focus our research efforts on better understanding how infant formula affects the balance of gut bacteria and their resistance to antibiotics. Long-term studies that follow babies from birth into childhood could give us important insights into how certain strains of bacteria become resistant to multiple drugs over time.

We also need to look into how specific ingredients in infant formula interact with gut bacteria and influence their ability to resist antibiotics. This could help us develop better formulas or dietary recommendations that support a healthier balance of gut bacteria and reduce the risk of antibiotic-resistant infections.

Furthermore, we should conduct studies to see if interventions like giving babies probiotics or changing the composition of infant formula can help counteract any negative effects on gut bacteria and antibiotic resistance. By finding ways to promote a strong and resilient gut bacteria balance early in life, we could potentially lower the chances of antibiotic-resistant infections and improve children's long-term health.

AUTHORSHIP CONTRIBUTIONS

Authors equally contributed to this work.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

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