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Detection Probiotic's DNA of *Lactobacillus paracasei* in Healthy Human Faeces

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Authors' contributions

This work was carried in collaboration between all authors who have designed the study. Author BMA has done all the laboratory tests, performed the statistical analysis and wrote the first draft of the manuscript. Author JW supervised the clinical part and author AAS supervised the laboratory tests. All authors read and approved the final study.

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ABSTRACT

Aims: To examine the DNA abundance of the probiotic bacteria (*Lactobacillus paracasei*) in the faeces of healthy adults after one month of its consumption and to determine the antimicrobial susceptibility profile of this bacteria.
Study Design: Thirty apparently healthy adults were examined for the presence of probiotic bacteria DNA in their faecal samples over a period of one month after one week of probiotic consumption.
Place and Duration: Department of Pathology and Microbiology, School of Medicine, The University of Jordan, Amman, Jordan, Between July 2017 and January 2018
Results: *L. paracasei* DNA detected in 90% of these adults within a week of probiotic consumption, whereas after stopping the probiotic consumption, *L. paracasei* DNA was detected only in 10% and 6% of the faecal samples after one and two weeks, respectively. Minimal side effects were recorded among these volunteers' adults. *L. paracasei* was susceptible to ampicillin, chloramphenicol, clindamycin, erythromycin, imipenem and piperacillin–tazobactam, intermediate

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susceptible to levofloxacin and ciprofloxacin and resistant to amikacin, aztreonam, ceftazidime, gentamicin, oxacillin, meropenem and vancomycin.
Conclusion: The consumption of probiotic *L. paracasei* for one week, resulted in a limited colonisation capacity in the human intestine, therefore, we recommend longer administration period. The susceptibility patterns of the probiotic bacteria *L. paracasei* should be considered when it will be administrated during antibiotic treatment.

Keywords: Probiotics; Lactobacillus paracasei; human faeces; antimicrobial susceptibility profile; antibiotics.

1. INTRODUCTION

Probiotics are increasingly used in the treatment of patients with gastrointestinal diseases and increasingly purchased by consumers without medical advice for health benefits [1-2].

The reasons for probiotic consumption were based on the assumption that it could produce beneficial health effects, particularly stimulating the immune response, competing with pathogens, producing vitamins, effectively reduce the duration and frequency of antibiotic-associated diarrhoea, constipation and other gastrointestinal diseases [2-3].

Probiotics are recognised to be safe because they have been consumed for a long time without observing serious side effects [3-4]. However, theoretically probiotics could result in some adverse effects; including overstimulation of the immune system, producing adverse metabolic activity, systemic infections and the contribution for antibiotics resistance factors as supported by its ability to transfer the resistance genes to other intestinal bacteria [5]. Therefore, it is recommended by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations to evaluate any new probiotics strain or safety by several methods [6].

There are specific criteria should be considered before consuming bacteria as a probiotic. These criteria have been found that certain strains of *Lactobacillus* and *Bifidobacteria* are the most beneficial bacteria to be used as probiotics. It should be also noted that even for the same species, probiotics bacteria could have different effects because each strain has its special biological characters such as its ability to colonise the intestine, the human body response to it and to which extent it can affect the composition of intestinal flora [7]. For example, glycosyltransferases, enzymes have an important role in glycoconjugates with variety of substance depending on their specificity [8], are essential for exopolysaccharide production, which in turn have immunomodulatory and antitumor effects, and this production varies greatly among the strains of the same species, [9] which depend on the genetic clusters like the special glycosyltransferases *welF* to -J genes that add the sugars to the exopolysaccharide in a sugar and glycosidic linkage-dependent manner [10]. Of note, previous studies of Ferrario, *et al.* and Balzaretti, *et al.* showed that the *welF* gene is unique to *L. paracasei* in the faecal sample [11,12].

One important factor which is still not well investigated is for how long probiotics should be consumed without causing any side effect. This study investigated the intestinal colonisation effect of one of the commonly used probiotics *Lactobacillus paracasei* in 30 voluntary healthy adults by detecting its DNA in faeces using PCR test as well as to determine the antimicrobial susceptibility profile of the organism.

2. MATERIAL AND METHODS

2.1 Study Design and Population

This prospective study included a total of 30 apparently healthy adult volunteers of both genders. All participants made a written consent to participate in this study and a short medical history was taken from each participant. Eligibility criteria included the following: a general good health, have not consumed antibiotic or prebiotic or probiotics in the last three months, did not have any digestive problem or chronic disease, did not have diarrhea or urinary tract infection in the last three months and have signed the consent forms.

The study protocol is implemented in accordance with The Declaration of Helsinki (2000). Ethical approval for the study protocol was obtained from the Institutional Review Boards at The Jordan University Hospital (Ref no. 2711/2017)

and the Deanship of Scientific Research at the University of Jordan. Additionally, a written informed consent was obtained from each volunteer to participate in this study according to the following instructions. Participants should follow their usual diets and each participant should give four fresh stool samples as follow: The first sample before the consumption of the probiotic, the second sample after one week of probiotic consumption, the third stool sample after one week of stopping the probiotic consumption; and the fourth stool sample after two weeks of stopping the probiotics.

2.2 Probiotic and Dosage

Participants took a probiotic capsule every day for one week (a total of seven capsules) in association with their habitual diet. Then all participants were informed to stop the probiotic consumption. The probiotic preparation (Enterolactis Plus) consisted of a gelatin capsule that contains at least 24 billion viable cells of the bacterial strain *L. paracasei* DG (*Lactobacillus paracasei* CNCM I-1572), Sofar Manufacture, Italy. All the Probiotics packages were checked for expiry date and kept at 4°C until the study start.

2.3 Volunteer Compliance

Compliance with the study protocol was assessed by interviewing the participants weekly and by measuring the number of faecal samples and the remaining probiotic tablets collected from each participant. All participants had been followed up during the four weeks of the study and they were informed to report to Dr. Jamal Wadi, MD any side effect that might appear with the use of the probiotic capsules.

2.4 Faecal Sample Processing

Faecal samples were collected using a sterile stool cup placed in a sterile plastic container. Participants were informed to keep the faecal samples at room temperature and delivered it to the lab no later than 24 h after the defecation according to the recommendation of Cardona et al. [13]. Immediately after delivery, stool samples were processed directly or stored at -80°C to be processed (within 2 days) for DNA extraction using QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions with slight modification as following: one glass bead (0.5 mm) was added to the faecal sample and the solution mixture in the first homogenisation step, and the vortex time was increased to 10 minutes to have full homogenisation of the sample [14]. The quantity and purity of DNA from each sample was determined using Nanodrop (Thermo-scientific, USA).

2.5 Detection and Quantification of *L. paracasei* DG in Faecal Samples

-PCR protocol was adopted for the detection of *L. paracasei* DG in the DNA of faecal samples targeting the *welF* gene, glycosyltransferase gene, with primers rtwelFf, 5′-TACTAAAGAAATTAGCTTTTGT-3′ and rtwelFr, 5′-AGTAATGTCTGCATCCTCCA-3′ with final PCR product of 625bp [11]. The gene was selected because the search in the GenBank nucleotide database revealed that this gene is unique to this strain. To standardise the PCR conditions a gradient PCR was initially performed. PCR amplifications were carried out in a final volume of 20µl containing 4 µl of the 5x FIREPol Master mix (Solis biodyne, Estonia) and 3.5µM of each primer. Samples were amplified with the following program: initial hold at 95°C for 5min, and 44 cycles at 95°C for 30 sec., 58ºC for 30 sec. and 1 min at 72ºC using the PCR thermocycler (Bioerxp cycler, China). PCR products were analysed using 2% agarose gels electrophoresis (Bio –Rad, USA). DNA from *L. paracasei* and positive sample were used as positive controls. Nuclease-free water and DNA from negative sample (from the first week) were used as negative controls. Bacterial DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA) according to manufactures instructions. PCR was performed at least in duplicate for each faecal sample.

-Real-Time PCR protocol was adopted to quantify *L. paracasei* DG in the DNA of faecal samples targeting the *welF* gene, glycosyltransferase gene, with primers *welFf*: 5'-GTCCCAAGATGACACAGTG-3' *welFr*: 5'-GACGGTATACGCACATCTG-3' [12] with final product of 171bp. To standardise the Real-Time PCR conditions a gradient Real-Time PCR was initially performed. Real-time PCR amplifications were carried out in a final volume 25 µL containing 12.5 µL of QuantiFast-SYBR-Green-PCR Master Mix (QIAGEN, Germany) and 1 µM of each primer. We used 100 ng of faecal DNA template in each reaction. Samples were amplified with the following programs: Initial hold at 95°C for 5min, and 40 cycles at 95°C for 10 sec. and 60ºC for 30 sec. using the Icycler IQ (Biorad, USA) PCR. To generate a standard

curve DNA was extracted from a pure culture of *L. paracasei* DG. Standard DNA of 100 ng bacterial DNA corresponding to 3.1×10^7 copy number was used to prepare three 3 ten-fold serial dilutions. Threshold cycle after the last serial dilution (0.1 ng bacterial DNA) considered lower than the limit of detection with a non-significant presentation of *L. paracasei* DG in the participants' faecal sample (*L. paracasei* DG DNA is less than 0.1 ng in 100 ng faecal DNA). Melting curves were analysed to confirm the specificity of the amplification products. Another confirmation was made on the Real-Time PCR product using 2% agarose gels electrophoresis (Bio –Rad, USA). Nuclease-free water and DNA from negative sample (from the first week) were used as negative controls. Analyses were run in duplicate in at least two independent experiments.

2.6 DNA Sequencing of *welF* gene of *Lactobacillus paracasei*

20 µl of the PCR product from *L. paracasei* DG pure culture (positive control) and 8 positive samples were sent to Macrogen company in South Korea for DNA purification and then DNA sequencing with (7ρ mole/µl) of each primer of *welF* gene. Obtained sequences were compared to available *Lactobacillus* sequences in the GenBank database, by using the Blast server. (http:// www.ncbi.nlm.nih.gov/blast/).

2.7 Antimicrobial Susceptibility Testing

The antimicrobial susceptibility pattern of *L. paracasei* DG was determined using the disc diffusion method for 15 antimicrobial discs (MAST Group Lt, UK, Table 2) according to the recommendation of Clinical Laboratory and Standards Institute /CLSI, 2016 [15]. *Escherichia coli* ATCC 35218 was used as a control for the susceptibility test and the tests were done in duplicate.

2.8 Statistical Analysis

Data generated from the study were analysed using Statistical Package for Social Sciences (SPSS version 20), symptoms were calculated for the categorical data by Fisher's exact test, to determine whether there are statistical differences with respect to gender or age groups. The level of significance was set at a p-value of 0.05 to test the hypothesis of no association, Fisher's exact test is used due to the small sample size.

3. RESULTS AND DISCUSSION

3.1 The Study Participants' Compliance and Reported Symptoms

Thirty apparently healthy adult human volunteers (13 females and 17 males) participated in our study, with age range of 18-77 years old and average of 30.8 years old. The participants had 100% compliance to the study protocol and therefore no participant has been excluded. During and following the probiotic *L. paracasei* DG consumption, all the participants have not reported any serious adverse effects, as shown in Table 1. This result is compatible with the findings of Balzaretti et al. who has used the same probiotic in his study [11]. Both studies confirmed the general safety of probiotic *L. paracasei* DG. However, it is important to note that 30% of the participants in our study noticed a transient change in their defecation frequency. This finding is considered a normal reaction because some strains of *Lactobacillus* species increase the bowel movement frequency in adults as reported also in other studies [16-17]. Furthermore, our study showed both constipation and diarrhea were reported in a few cases without significance, whereas fever was not observed in any case during the period of probiotic consumption. These findings are similar to the study carried out by Ojetti et al. [16].

3.2 Presence of *Lactobacillus paracasei* in Participants' Faecal Samples

The present study demonstrated that *L. paracasei* was not detectable in all faecal samples before the probiotic consumption. However, one week consumption of the probiotic was enough to detect the *L. paracasei* in 90 % of the participants' faecal samples using PCR (Fig. 1). However, we did not find significant persistence of the *L. paracasei* in the faecal samples collected after the probiotic consumption. Specifically, the *L. paracasei* concentrations were below the Real-time PCR detection limit. (Fig. 3). Of note, Ct values as well as melting curve analysis show compatible presence of the *L. paracasei* in the faecal samples and the DNA sequence of the *welF* gene of *Lactobacillus paracasei* were confirmed to hit against *L. paracasei* EPS-b region, strain DG (CNCM I-1572) with an identity homology of 99 %.

This result is compatible with other studies which indicated that high doses of probiotics are

needed to increase a significant number of specific bacteria and consequently its DNA in the faeces [18]. Of note, the probiotic bacteria was detectable in the faecal samples of all 18-30 years old participants in comparison to 72 % of ≥30 years old participants with no significant difference between age or gender groups (P-value= 0.6957). After ceasing the probiotic consumption for one and two weeks, *L. paracasei* was detected only in 10 % and 6 % of the participants' faecal samples, respectively. Notably, after stopping the probiotic, *L. paracasei* was detected only in the faecal samples obtained from female participants. (Fig. 2). These data are in agreement with other studies [7,11,19]. For example, the recent study of Balzaretti, *et al.,* [11] have used the same strain of *L. paracasei* for short duration and compared it with other strains in 8 healthy adults. Their study found that the two probiotic bacteria cannot be detectable in the stool after 7 ± 2 days of stopping the consumption of the probiotics. The study of Tuohy et al. [7] used a different strain of *Lactobacillus* which was delivered in fermented milk in large doses, was consumed by 10 healthy adults twice daily for 3 weeks. Their study has shown that the probiotic bacterial counts were significant and high during the period of consumption the fermented milk product, but no significant difference in bacterial counts found in stool samples between the days 7, 14, 21 after consuming the probiotic. Additionally, the probiotic bacterial counts in stool after one week of stopping it consumption were less or not detected in all the volunteers. All these studies have confirmed that the probiotic dose of *L. paracasei* strain should be high to have a significant presence in the intestine as shown in our study. However, the study of Ferrario, *et al.,* [12] reported that the impact of probiotics is strictly depends on the initial characteristics of the intestinal microbial ecosystem in each person. In addition, there is no clear recommendation about the duration of consumption of *L. paracasei* product, and this issue is still not well established in other studies. Moreover, the results of our study and others cannot be generalised because each bacteria species and strain may induce different effects to colonise and persist for short or long time in the host gut [7].

Table 1. Shows volunteers gender and age groups and the symptoms reported during probiotic consumption

Age group	Male	Female	Total	
18-30 years old	10	9	19	
≥30 years old	7	4	11	
Gastrointestinal illness	Not reported	Non reported		
Total	17	13	30	
Symptoms reported during probiotic consumption	No. (%) Positive	No. (%) positive	No. (%) Positive	P-value to gender comparison
Faecal mal-digestion	Nil	2(15)	2(6)	0.1793
Diarrhea	Nil	1 (7)	1 (3)	0.4333
Constipation	Nil	3 (23)	3 (10)	0.0704
Change in defecation frequency	6 (35)	3 (23)	9 (30)	0.3772

**P-value was not significant in relation to the gender groups*

Table 2. The antimicrobial susceptibility pattern of the probiotic bacteria strain *Lactobacillus paracasei* DG, CNCM I-1572

Antibiotic (Disk concentration/ug)	Result*	Antibiotic (disk concentration/ug)	Result*
Amikacin(30)	R	Gentamicin(30)	R
Ampicillin(5)	S	Imipenem(10)	S
Aztreonam(30)	R	Oxacillin(1)	R
Ceftazidime(30)	R	piperacillin – tazobactam (110)	S
Ciprofloxacin(5)	I	Levofloxacin(5)	I
Chloramphenicol(30)	S	Meropenem(10)	R
Clindamycin(2)	S	Vancomycin(30)	R
Erythromycin(15)	S		

**R=Resistant; S= Susceptible; I= intermediate susceptible*

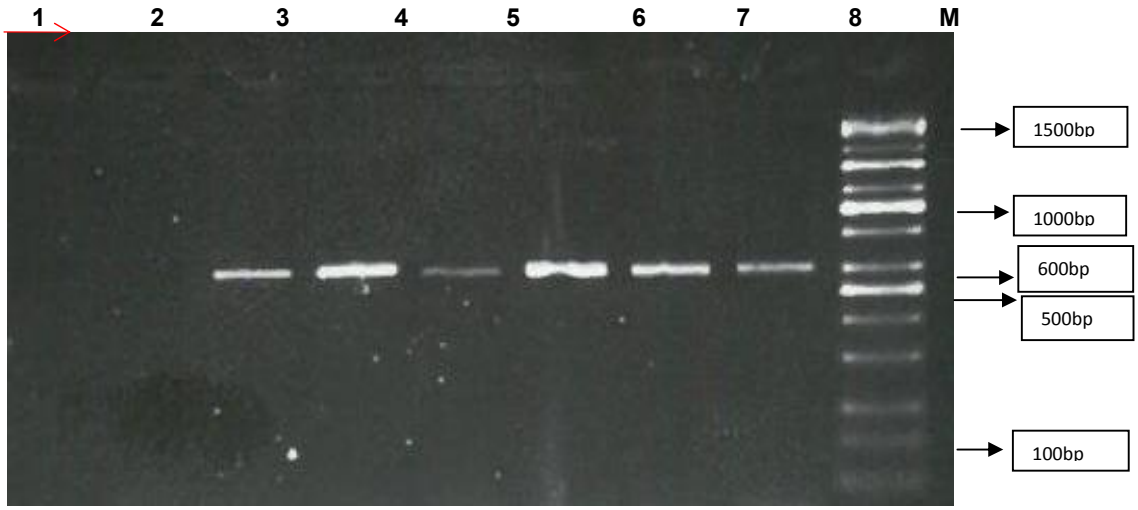


Fig. 1. Agarose gel electrophoresis of the amplified *welF* gene specific sequence for *Lactobacillus paracasei*. The final PCR product was 625 bp by uniplex PCR. lane 1 negative nuclease free water, lane 2 negative sample, lane 3-4 positive controls from *L. paracasei* pure culture and positive sample respectively, Lanes (5-8) positive DNA of *L. paracasei* in the faecal samples of the volunteers. lane M: 100-bp DNA ladder

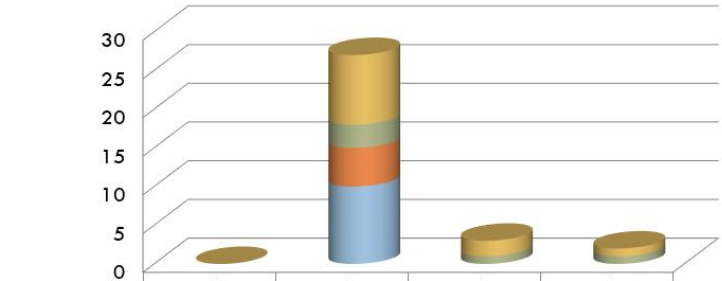
3.4 Antimicrobial Susceptibility of *Lactobacillus paracasei*

The result of this study showed that the probiotic (Enterolactis plus) bacteria *L. paracasei* was resistant to the following antibiotics: amikacin, aztreonam, ceftazidime, gentamicin, oxacillin, meropenem and vancomycin and susceptible to ampicillin, chloramphenicol, clindamycin, erythromycin, imipenem and pipracillin-tazobactam, while intermediate susceptible to ciprofloxacin, and levofloxacin (Tabel 2).

The genus *Lactobacillus* is the largest group of the lactic acid bacteria which are widely used as probiotic in many food items especially in fermented dairy products. This genus is usually susceptible to penicillins, but are more resistant to cephalosporins. However, many *Lactobacillus* species are intrinsically resistant to vancomycin, and most antibiotics which inhibit nucleic acid synthesis may inhibit most of *Lactobacillus* species. Additionally, *Lactobacillus* species are commonly resistant to aminoglycosides and other inhibitors of protein synthesis, while their resistance to other antibiotics varies greatly among *Lactobacilli* strains [20]. Therefore, and not surprising the study of Li et al. [21] examined the antimicrobial susceptibility pattern of other probiotics bacteria of the same genus of *Lactobacillus* and has found a different antibiotics susceptibility profile. These results conclude that the consumption of *L. paracasei* should be combined only with those antibiotics which will not inhibit the survival of the organism in the intestinal tract of the patient. Therefore, the antimicrobial susceptibility profile for each used probiotic product should be provided to let the physician know if the probiotic can be combined with the prescribed antibiotic.

It is well established that antibiotic administration has adverse effects on gut flora by reducing the abundance and diversity of beneficial commensal microbiota [22]. Antibiotics consumption may also increase the presence of resistant bacterial strains in the intestinal flora of human and animal over the time [22-24]. In addition, usage of antibiotics, prebiotics and probiotics can modify the gut microbiota of humans and domestic animals [25]. It has been reported that *Lactobacillus* species carry frequently plasmids and transposons, and some of their resistance determinants have been found to be transferred *in vitro* between strains of *Lactobacillus* and other Gram-positive bacteria, including food pathogens such as *Staphylococcus aureus* [20]. Moreover, these resistance determinants may be efficiently transferred under selective pressure of antibiotics treatment in the intestines of the host [26].

In addition, it is not known if this *L. paracasei* strain carries antimicrobial resistance genes which can be transferable in the human gut? especially, during antibiotic treatment. Therefore, further investigations should be done to answer this important question.



	week 1	week 2	week 3	week 4
female <30	0	9	2	1
female ≥30	0	3	1	1
male ≥30	0	5	0	0
male <30	0	10	0	0

Fig. 2. PCR results for the detection of the *L. paracasei* in 30 participants' faecal samples before probiotic consumption week 1, after one week of probiotic consumption week 2, after one and two week of stopping the probiotic week 3 and 4, respectively

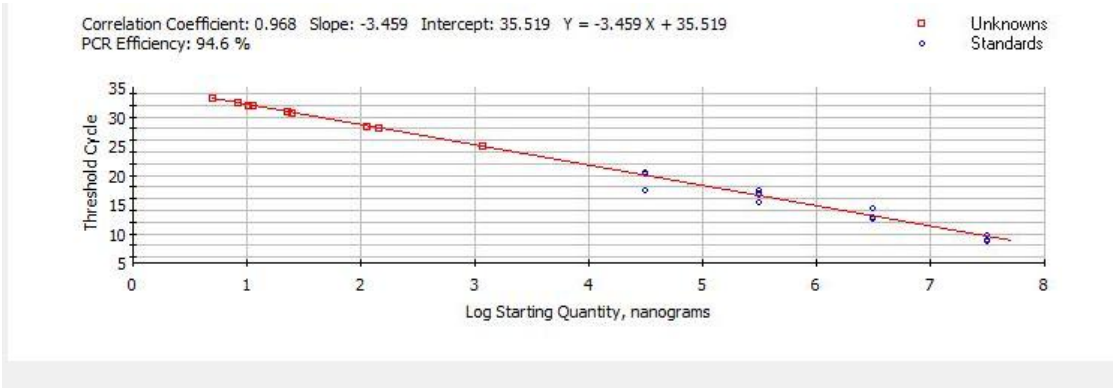


Fig 3. Real-Time PCR Standard curve for the detection of *L. paracasei* in participants' faecal samples, which shows the standards and the positive samples that were lower than the detection limit

4. CONCLUSION

The low detection of probiotic's DNA of *Lactobacillus paracasei* in faeces of volunteers after consumption of one capsule/day of the bacteria for one week shows its limited colonisation capacity in the human intestine.

CONSENT

All participants made a written consent to participate in this study and a short medical history was taken from each participant.

ETHICAL DISCLAIMER

The study protocol is implemented in accordance with The Declaration of Helsinki (2000). Ethical approval for the study protocol was obtained from the Institutional Review Boards at The Jordan University Hospital (Ref no. 2711/2017) and the Deanship of Scientific Research at the University of Jordan.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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