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ORIGINAL ARTICLE

Comparison of the prevalence of bacteriocin encoding genes in *Lactobacillus* spp. isolated from fecal samples of healthy volunteers, IBD-patient and IBD-recovered

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ABSTRACT

Background and Objectives: Bacteriocins are antimicrobial peptides produced by many genera of bacteria especially *Lactobacillus* spp. against many pathogens, adapt bacterial composition in the gut and inhibit dysbiosis that can lead to inflammation disorders like inflammatory bowel disease (IBD). The aim of this study was to compare the prevalence of bacteriocin genes in health, IBD disease and recovery conditions.
Materials and Methods: In this survey 115 *Lactobacillus* spp. from 58 fecal samples of three different groups were evaluated. Comparison of the presence of bacteriocin genes in different groups were assayed by purified samples and PCR method, followed by statistical analysis to identify the effect of inflammation in the proportion of *Lactobacillus* spp. and presence of their bacteriocin genomes.
Results: Of 115 *Lactobacillus* spp. 60% of samples had positive bacteriocin-encoding genes which included: *gassericin*-A 29.56%, *acidocin* 15.65%, *plantaricin*-NC8 18.26%, *plantaricin*-S 13.04%, *lactacin*-F 9.5%, *sakacin*-P 6.08% and *gassericin*-T 6.08%. Results indicated that the percentage of positive bacteriocin genes were much more in healthy volunteer and IBD-recovered in comparison to IBD-patients which showed the effect of inflammation in the presence of bacteriocin genes.
Conclusion: The results obtained in this study demonstrated that the presence of bacteriocin genes can be related to health and disease states and inflammatory disease affected the prevalence of bacteriocin-encoding genes. This approach can help to identify bacterial functions that can be targeted in future concepts of IBD therapy.

Keywords: *Lactobacillus*; Antimicrobial peptides; Dysbiosis; Bacteriocins; Inflammatory bowel disease

INTRODUCTION

The gastrointestinal tract (GIT) is home to extensive and various symbiotic microorganisms. The GIT microbiota pattern has a crucial role in host health. Disruption in this pattern can lead to immune system dysregulation and impact on normal homeostasis (1). Changing the microbial pattern can cause inflammatory diseases like inflammatory bowel disease (IBD). IBD-patients suffer from dysbiosis, which is defined as a microbial pattern disorder and characterized by reduction in frequency and quantities of efficient in-

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testine bacteria (2). Clinical symptoms of IBD are identified by two types: Ulcerative Colitis (UC) and Crohn's Disease (CD). UC leads to inflammation of the whole gastrointestinal tract, while CD influences colon and rectum zones (3, 4). IBD is an essential global health issue that mainly affects young adults and increases morbidity and the risks of developing cancers. The etiology of IBD is related to several factors such as genetics or environmental factors but it is not obviously clear (5).

At the dysbiosis regards, recently, manipulation of bacterial genera in the gut by using probiotic strains -live microorganisms, which when administered in adequate amounts, confer a health benefit on the host- has been interested in the management of IBD (2). In this context, the main goal of using the probiotic is protecting of beneficial bacteria population in the gut that some studies showed the bacterial composition and functions changes in some disorders like IBD (6). One of the main roles of the bacterial species in the gut, in addition to consumption improvement, vitamin production and immune system manipulation, is the prevention of bacterial and viral infections. Changes in the gut bacterial composition by increasing Gram-negative species could affect the IBD-patients' condition and illness progression (7).

Lactic acid bacteria (LAB) especially *Lactobacillus* spp. release various agents to compete against other species and pathogens by limiting them to attach cell sites (8). One of the effective agents that impacted mainly on pathogens and bounded their growth is bacteriocin which is defined as antimicrobial peptides with antibacterial activity (9). Therefore, producing bacteriocin in *Lactobacillus* strains is an important probiotic characteristic that can demonstrate their potential to use them instead of antibiotics with lesser side effects (10).

Bacteriocins have been tested as safe and effective alternative molecules over the currently used chemotherapeutic agents. Thus, being an important clinical significance, its screening and recovery methods along with its application are poorly described (11). According to recent information on functional changes in commensal bacterial species in the gut, in this study we tried to investigate the changes in *Lactobacillus* species population and bacteriocins production as one of the more important probiotic strains, in new cases of IBD and symptomatically managed patients in comparison to healthy people. The purpose of this study was to compare the prevalence of bacteriocin encoding genes in *Lactobacillus* spp. isolated from healthy volunteers, IBD-patients and IBD-recovered.

MATERIALS AND METHODS

Sample collection. Fecal samples were obtained from the collection of the gastrointestinal ward of Firoozgar Hospital (Tehran, Iran). Samples divided into two groups:

1. Healthy volunteers: without gastrointestinal diseases, IBD, and recent antibiotic consumption.

2. IBD patients, which were in two groups: IBD-recovered patients: The drugs used to treat the IBD patients were 5-aminosalicylic acid or sulphasalazine in combination with a corticosteroid together with azathioprine, cyclosporin A, anti-tumor necrosis factor (TNF)-α monoclonal antibodies (infliximab and adalimumab) (12). New IBD patients: who suffered from diarrhea, occult in faces, stomachache and were diagnosed as IBD-patients with UC or CD types of disease. The healthy group consisted of 16 females and 19 males, and the IBD groups have consisted of 11 females and 12 males (13, 14) (age group: 18-60). We worked on 550 bacterial strains isolated from the fecal samples at Pasture Institute of Iran.

Screening isolated bacteria. Isolates were cultured on De Man, Rogosa, Sharpe (MRS) broth (Fisher Scientific, Canada) for 24 hours at 37°C under anaerobic condition. Gram staining method was used to select the Gram-positive bacteria based on morphology and color. Moreover, a catalase test was done to select catalase-negative species that could be candidates as *Lactobacillus* spp.

Probiotic tests. Probiotic bacteria should remain alive under the acidic condition of the stomach and the toxicity of bile. Resistant to acid and bile tests were done by diluted bacterial suspension at different times in MRS broth (Fisher Scientific, Canada) containing acid. A 100 µl of bacterial suspension was inoculated into sterile MRS broth adjusted to pH 2.0 with HCl for acidity resistance test and with 0.30% ox bile (Oxoid) to determine bile salts resistance of the strains. After overnight incubation anaerobically at 37°C, bacterial colonies were counted and compared at different times, and they were identified by monitored hourly for growth at 620 nm (15, 16).

DNA extraction. Resistance to acid and bile salts was used to select the strains for DNA extraction. DNA was extracted from pure cultures grown on MRS agar. Isolates were diluted in MRS broth (24 h, anaerobically) and centrifuged (8000 rpm, 10 min) to detect precipitate. DNA isolation was done using an extraction kit (GeneAll, Germany), and its concentration was monitored by NanoDrop (Thermo Scientific, USA) at wavelengths of 260 and 280 nm.

PCR to confirm *Lactobacillus* spp. PCR was performed using reverse and forward primers designed by McOrist et al. (2002) F:5´-TGGAAACAGGTGCTAATACCG-3´, R:5´CCATTGTGGAAGATTCCC-3´ (15) to detect *Lactobacillus* isolates. 25 µl volume considered as PCR reaction by 12.5 µl Master Mix (Amplicon, Denmark), 9.5 µl DDW, 1 µl forward primer, 1 µl reverse primer, and 1 µl template DNA. A thermocycler machine (Eppendorf Biotech Company, Hamburg, Germany) was used for amplification. The condition was initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec followed by the final extension at 72°C for 7 min.

PCR for bacteriocin encoding genes detection. PCR reaction for 25 µl volume was prepared using 12.5 µl Master Mix (Amplicon, Denmark), 9.5 µl DDW, 1 µl template DNA, 1 µl forward primer, and 1 µl reverse primer (Table 1). Bacteriocin genes were assayed by PCR thermocycler (Eppendorf Biotech Company, Hamburg, Germany), and all the results were checked by gel electrophoresis. The PCR test was performed under the following conditions: Initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. The final extension step was performed at 72°C for 10 min.

Statistical analysis. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance was generated for the healthy group compared with IBD recovered and IBD-patients by one-way analysis of variance (ANOVA) with Fisher's LSD test. T-test used for comparison between healthy and total IBD. SPSS statistic software package was used (version 20. 0, IBM Corporation, New York, NY, USA). *P*-value of $P<0.05$ were considered significant.

RESULTS

From 550 isolated bacteria, 115 isolates were rod-shaped, Gram-positive, catalase-negative, and resistant to acid and bile (probiotic features); all isolates belonged to the *Lactobacillus* as confirmed by PCR. In the healthy volunteer group (n=35), 87 *Lactobacillus* strains were isolated. From the patient group with IBD (n=23, 5 individuals with Crohn's disease, and 18 with Ulcerative colitis), 28 *Lactobacillus* strains were isolated. This group included new cases with IBD (n=9) and IBD-recovered one (n=14). From new

Table 1. Primers used for PCR

Genes	Primers	Sequences	TM°C	Product length (bp)	Ref.
sakacin p	*sakpF*	ATGGAAAAGTTTATTGAATTA	48	186	(17)
	sakpR	TTATTTATTCCAGCCAGCGTT	55		
acidocin	*acdAF*	GTTGCAGGATCARGTG	49.26	84	(18)
	acdAR	TGTTGCAGCTCCGTTA	52.62		
gassericin A	*gaaF*	GAACAGGTGCACTAATCGGT	57.91	822	(18)
	gaaR	CAGCTAAGTTAGAAGGGGCT	56.62		
gassericin T	*gatF*	GGTGGGAGAAATAATTGGGC	56.43	710	(18)
	gatR	CCTATTACAAACGATATGGCC	54.23		
plantaricin S	*PlsF*	GCCTTACCAGCGTAATGCCC	61.44	320	(19)
	PlsR	CTGGTGATGCAATCGTTAGTTT	57.31		
plantaricin NC8	*PLNC8F*	GGTCTGCGTATAAGCATCGC	59.22	207	(20)
	PLNC8R	AAATTGAACATATGGGTGCTTTAAATTCC	56.23		
lactacin F	*lafAF*	AGTCGTTGTTGGTGGAAGAAAT	58.45	184	(21)
	lafAR	TCTTATCTTGCCAAAACCACCT	57.89		

cases with IBD, 3 *Lactobacillus* strains and 25 *Lactobacillus* strains were isolated from recovered one. Results from 23 IBD patients, 28 *Lactobacillus* strains were isolated which 89% (25/28) of them were from the IBD-recovered group, and 10.71% (3/28) of them were from new cases with IBD.

From 115 *Lactobacillus* strains, 60% of isolate contained bacteriocin genes which included: *gassericin*-A 29.56%, *acidocin* 15.65%, *plantaricin*-NC8 18.26%, *plantaricin*-S 13.04%, *lactacin*-F 9.56%, *sakacin*-P 6.08%, and *gassericin*-T 6.08% (Table 2). Of 113 detected (positive) bacteriocin genes, 57% were female, and 42% were male. Moreover, in patients (IBD and recovered), UC positives were 59% (16/27), while CD positives were 40.74% (11/27) which were mostly from the IBD-recovered group. Subsequently, much more detected bacteriocin gene prevalence was in females than males and in individuals with Crohn's disease than recovered. Most *Lactobacillus* strains had bacteriocin genes, but percentage frequency each gene was low (Table 2).

Comparison of detected bacteriocin genes in three different groups (healthy, cured-IBD and IBD) revealed that the prevalence of bacteriocin genes (*gaaA, acidocin, lacF*) in the healthy and IBD-recovered groups were more than IBD group (Fig. 1). In some other genes, in which IBD positives have been shown more than other groups (Fig. 1), the number of positive samples was much less than healthy and recovered groups (Table 2). It showed the low frequency of *Lactobacillus* spp. in IBD fecal samples and the relation between inflammation disease and the prevalence of bacteriocin encoding genes. Furthermore, compared to healthy individuals and total IBD (recovered and patients), *acidocin, plNC8,* and *plS* positive genes were more in the healthy group (Fig. 2). However, even in the total IBD group, which have been shown more positive bacteriocin genes (*sakP, gaaA, gasT, lacF*) than the healthy group, most *Lactobacillus* strains were isolated from recovery individuals (Fig. 2 and Table 2) that may confirm the beneficial effect of the presence of bacteriocin genes in health condition. Comparison of all detected genes based on percentage of each gene in health, disease and recovered conditions showed in Fig. 3.

DISCUSSION

Inflammatory bowel disease (IBD) is a worldwide crucial health issue and the prevalence of inflammatory disease increases globally, especially in developing countries (5, 22). IBD has no age limit and can effect on children or adults, while the cause of this inflammation is ambiguous (3, 4). Evidence indicated a crucial role of commensal gut bacteria in IBD and demonstrated that changes in intestinal microbiota composition could lead to UC and CD disorder. As a result, reconstitution with commensal gut bacteria is a key factor to the etiology of inflammation disease development (7). Some strains belong to *Lactobacillus* spp. *Bifidobacterium* spp. *Streptococcus salivarius, Saccharomyces boulardii, Clostridium butyricum, Ruminococci* and *Escherichia coli* seems to be beneficial, while alteration in gut microbiome with some other strains such as *Bacteroides* spp. *Enterococcis faecalis, Enterobacter cloacae,* intestinal *Heliobacter* spp. *Fusobacterium* spp. adherent/

Table 2. Positive bacteriocins genes separately sorted

	sak P	***gaaA***	***acidocin***	***plS***	***plNC8***	***lac F***	***gas T***	**Total**
Healthy (n=87)	4.59% (n=4)	22.98% (n=24)	19.54% (n=17)	13.79% (n=12)	20.68% (n=18)	8.04% (n=7)	4.59% (n=4)	86 genes 54 samples (62.06%)
CIBD (n=25)	8% (n=2)	40% (n=10)	4% (n=1)	8% (n=2)	8% (n=2)	16% (n=4)	8% (n=2)	23 genes 15 samples (60%)
IBD (n=3)	33.33% (n=1)	0	0	33.33% (n=1)	33.33% (n=1)	0	33.33% (n=1)	4 genes 1 sample (33.33%)
Total (n=115)	6.08% (n=7)	29.56% (n=34)	15.65% (n=18)	13.04 (n=15)	18.26% (n=21)	9.56 (n=11)	6.08 (n=7)	113 genes 70 samples (60.86%)

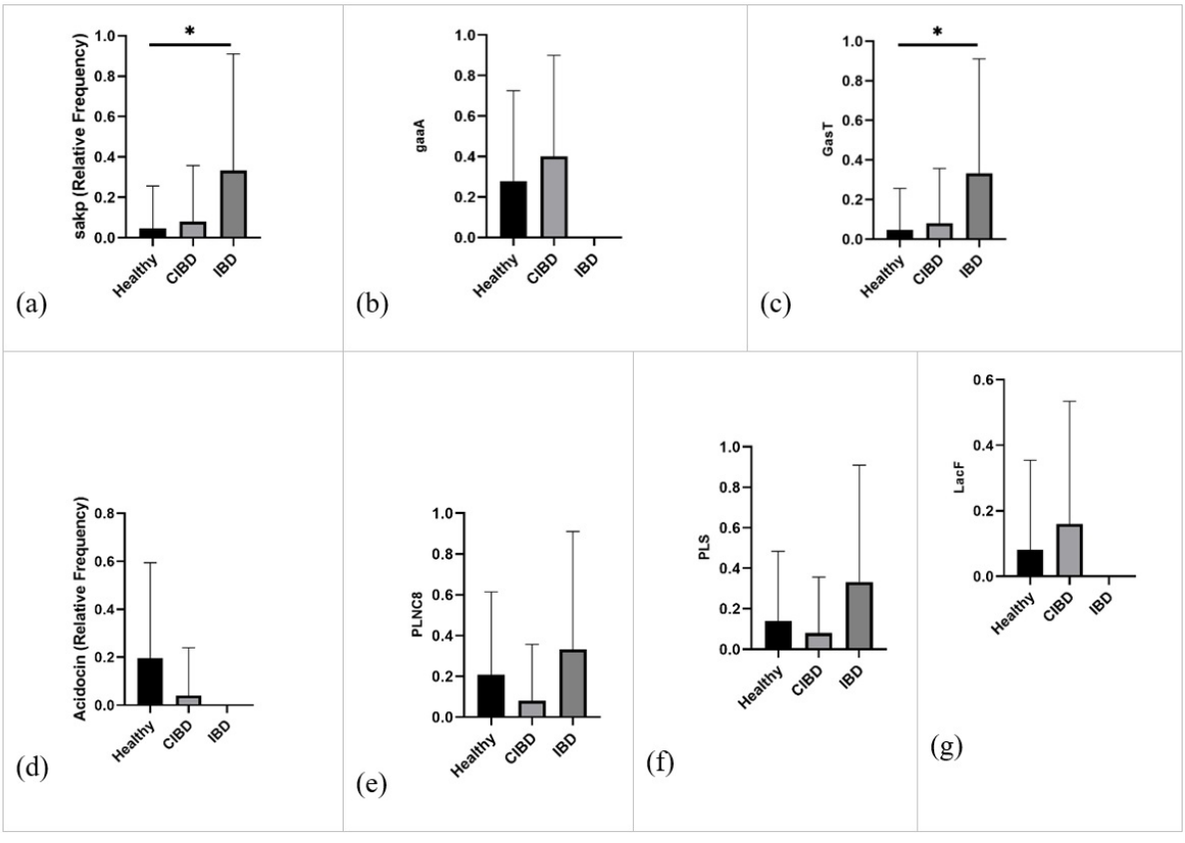

Fig. 1. Comparison of positive bacteriocin encoding genes (*sakP, gaaA, gasT, acidocin, plNC8, plS, lacF*) in three different groups (healthy, cured-IBD and IBD) (a-g). The one-way analysis of variance (ANOVA) is used to determine statistically significant differences. Data in *sakacin P* and *gassericin T* genes between healthy and IBD groups by Fisher`s LSD test are means ± SEM. **P*<0.05. Relative Frequency is based on ratio of detected samples to total samples.

invasive *Escherichia coli* strains, *Eubacterium* and *Peptostreptococcus* spp. have been associated with IBD (23, 24).

Studies have shown that disbalance in the gut microbiome impact on mucosa and tissue of intestine is caused by increasing harmful bacteria like *Escherichia coli* and decreasing protective ones like *Bifidobacterium* and *Lactobacillus* species (25). The hypothesis focused on regulating GI composition can lead to therapeutic results in IBD (26). Using chemical treatments or other therapeutic agents like probiotics, inflammatory diseases like IBD can heal and reveal inflammatory symptoms (12). As indicated in recent researches, bacteria composition especially *Lactobacillus* strains composition altered in health and disease conditions (25). *Lactobacillus* strains are potential bacteriocin producers that can modulate immune systems and host health by releasing these antimicrobial agents to compete against pathogens and protect their established niches (27).

In this survey the prevalence of some common and novel bacteriocin genes such as *sakacin* P, *plantaricin*, *lactacin* F and *gassericin* (A & T) were evaluated. These genes are mostly located on the chromosome, the main part of bacterial genomes (18). Moreover, we compared three groups, including healthy volunteers, IBD-recovered and IBD-patients to see the effect of bacterial composition in bacteriocin presences which indicated the different prevalence of *Lactobacillus* spp. in health, recovered and disease states. Furthermore, we mention that some of our genes were identified as novel bacteriocin genes like *gassericin* T which is produced from *Lactobacillus gasseri* LA327 (8). This bacteriocinigenic strain produced two kinds of class IIb bacteriocin structural genes identified as *gassericin* T (GT) and *acidocin* LF221A (Acd LF221A). The *acidocin* LF221 A and *acidocin* LF221 B were predicted to be members of the two-component class II bacteriocins, where Acidocin LF221 A appears to be a novel bacteriocin.

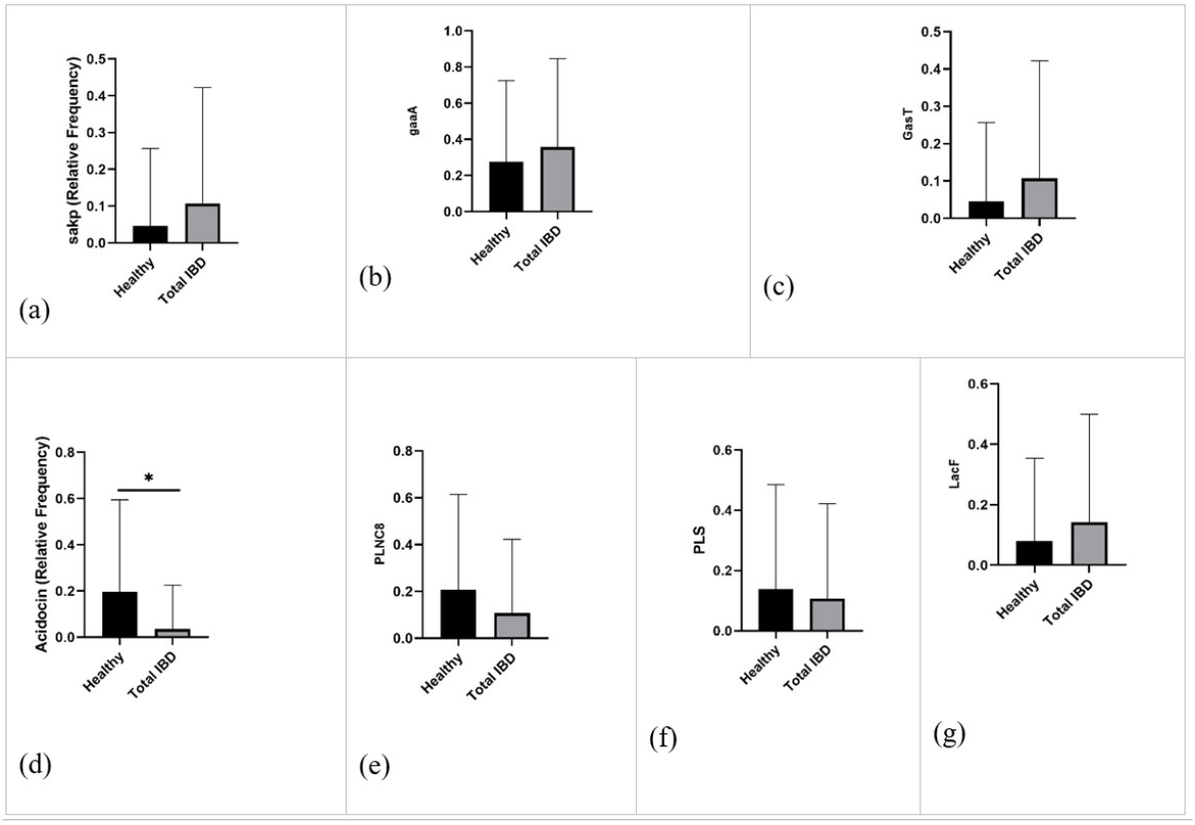

Fig. 2. Comparison of positive bacteriocin encoding genes (*sakP, gaaA, gasT, acidocin, plNC8, plS, lacF*) in two different groups (healthy and total IBD (patients and recovered)) (a-g). T-test is used to determine statistically significant differences. Data in *acidocin* gene between healthy and total IBD groups are means ± SEM. **P*<0.05. Relative Frequency is based on ratio of detected samples to total samples.

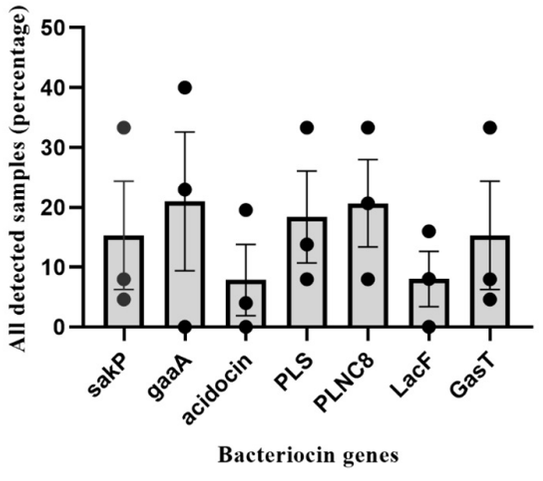

Fig. 3. The prevalence of all detected bacteriocin genes in one frame. The percentage of positive healthy, IBD, and CIBD *Lactobacillus* isolates for each bacteriocin gene has been shown by black circles in each bar based on Table 2.

These two types produced by *Lactobacillus gasseri* LF221 were isolated from the feces of the child. The results of DNA sequencing revealed that two-peptide (class IIb) bacteriocins exhibited the maximum activity through the synergy of two components, and their antimicrobial spectra were known to be relatively wide. Moreover, *L. gasseri* LF221 was developed as a potential probiotic strain and a food preservative (8, 28). These data demonstrated the effect of bacteriocins in health conditions.

Most studies just focused on the presence of bacteriocin genomes but the comparison between health and disease conditions had been missed which it was considered in our survey. For instance, Stoyancheva G et al. (2014) conducted a study on bacteriocin production of *Lactobacillus* in the vagina (18). In their survey, 17 new samples were isolated and the pure chromosomal DNA was isolated. Polymerase chain reaction was performed and the PCR products were visualized. All isolates belonging to species of *Lactobacillus* were tested for the prevalence of genes

encoding the bacteriocins: *gassericin* A, *gassericin* T and *acidocin* lF221A, but just the presence of bacteriocin genes in healthy samples were determined, while in our study comparison of them in health and disease conditions were considered.

From 115 resistance *Lactobacillus* spp. obtained from stool samples of 35 healthy individuals and 23 patients with IBD, 87 isolates with probiotic features were isolated from healthy group and 28 isolates from the IBD groups, in which 3 isolates belonged to patients with newly diagnosed and 25 to patients with recovered IBD. Detected more becteriocin genes in healthy and recovered groups indicated the efficient impact of bacteriocin properties although, size sample limitation due to low power may lead to non-significant differences in some groups' comparison that can solve by considering more size samples in future similar studies. Our results on detecting bacteriocin genes, showed that approximately 60% of all *Lactobacillus* isolates had bacteriocin genes but the frequency in each gene was low individually, similar to some evidence in other approaches. In research that Bibalan et al. (2017) examined bacteriocin genes of *Lactobacillus* strains isolated from feces of healthy individuals, 72 *Lactobacillus* species were obtained from 434 lactic acid bacteria (LAB) strains. Approximately 40% of isolates had antimicrobial activity against organisms and just 17.4% of them were active against indicator bacteria. The frequencies of genes were *gassericin* A 5 (6.9%), *plantaricin* S 3 (4.1%) and 5 (6.9%) *laf*. They found that of 40% positive *Lactobacillus* isolates for bacteriocin phenotype test (antibacterial activity), Just 6% of them had bacteriocin genes (29). Furthermore, the immuno-modulation role of bacteriocins in inflammatory diseases had confirmed. The bacteriocin produced by *L. plantarum* WCFS1, *plantaricin*, has been shown to be pivotal in the synthesis of the anti-inflammatory cytokine IL-10 and generally have anti-inflammatory properties (30).

Studies supported our results on the beneficial effect of bacteriocin in health and the lack of its production in inflammatory disease. Yin et al. (2018) studied on bacteriocin biosynthesis related to anti-inflammatory characteristics of *Lactobacillus* on a mouse model with acute IBD which bacteriocin genes of two different mutants of *Lactobacillus* strains were knocked out. Mice that feed by these two mutants of *Lactobacillus* had not been shown any protection against colitis. These findings indicated the beneficial effects of plantaricin production of *Lactobacillus* in the gut health system (31). It showed the essential impact of the presence of bacteriocin genes in health and bacteriocin deficient in inflammation as demonstrated in the present study.

CONCLUSION

Studies on the prevalence of different bacteriocin genes in inflammatory diseases are not expanded yet. This survey focused on the presence of different bacteriocin genes and compared them with each other in different conditions defined as health, disease and recovered states to identify the effect of these crucial peptides in healing inflammation. This investigation revealed that bacteriocin genes of *Lactobacillus* spp. are key factors that lead to human intestine health. Moreover, recovery after an inflammatory disease like IBD is related to bacterial composition and the presence of bacteriocin encoding genes. This approach can help to confirm the role of bacterial functions that can be targeted in future concepts of IBD therapy.

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