ORIGINAL ARTICLE

AG-2018-47
dx.doi.org/10.1590/S0004-2803.201800000-70

Honey-derived *Lactobacillus rhamnosus* alleviates *Helicobacter pylori*-induced gastro-intestinal infection and gastric inflammation in C57BL/6 mice: an immuno-histologic study

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Received 8/4/2018
Accepted 30/5/2018

ABSTRACT – Background – *Helicobacter pylori* (*H. pylori*) has been introduced by since 1983 by Marshal and Warren to play the main role in the pathophysiology of gastritis and gastric ulcers. Almost half of the world population1 is infected by *H. pylori*. Current therapeutic regimen against *H. pylori* includes the use of a proton pump inhibitor plus two or more antibiotics. However, the efficacy of this regimen is decreasing mainly due to antibiotic resistance and side effects of medications. This fact has resulted in public interest in other therapeutic options and the role of probiotics merits special attention in this regard. **Objective** – This study aims to evaluate the efficacy of honey-derived *Lactobacillus rhamnosus* on *H. pylori*-induced gastric inflammation and gastro-intestinal infection in C57BL/6 Mice. **Methods** – The 24 C57BL/6 Mice were randomly divided into three groups of eight mice each. All the mice were fed with 1cc suspension containing $5*10^{10}$ CFU/ mL of ATCC43504 strains of *H. pylori* for 3 consecutive days, twice daily via polyethylene gavage tubes. At the end of 4th week, infection with *H. pylori* was confirmed with stool Ag (ELISA) and following sacrifice of one mouse from each group, histopathologic study confirmed gastritis. The groups were subjected to different therapies as stated, 1: without Bismuth (Bi), Omeprazole (Om) and *L. rhamnosus* prescription, 2: Bi, Om and Clarithromycin (Cl) and 3: Bi, Om plus 1cc of suspension of 10^9 CFU/mL of *L. rhamnosus*. After 2 weeks, the stool was analyzed for Ag and the mice were sacrificed for evaluation of histopathologic changes. **Results** – Treatment with *L. rhamnosus* group provided Zero titer of stool Ag and was associated with improved gastric inflammation in all subjects, similar to the clarithromycin group. **Conclusion** – Honey-derived *L. rhamnosus* probiotics provides similar results as clarithromycin in terms of improvement of *H. pylori* infection and gastritis in C57BL/6 Mice model, without its cons of antibiotic resistance.

HEADINGS – *Helicobacter pylori*. *Lactobacillus rhamnosus*. Stomach ulcer. Inbred C57BL mice. Honey.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram negative microaerophilic bacillus which is motile and pathogenic due to possessing flagella. *H. pylori* infection is pandemic and its prevalence ranges from 41.35% to 72.3% in different countries, worldwide[1,2]. In many developing countries, the infection rate rises to 80% to 90% of the adult population. Prevalence of infection is variable between the countries and between the races and ethnicities in a certain country and is dependent on socioeconomic status of population residing in that environment.

Barry Warren and Robin Marshall were the first to isolate *H. pylori* in 1983 which resulted in their achievement of Nobel Prize in physiology for their pioneering work on *H. pylori* and its role in gastritis and peptic ulcer disease. In 1994 the International Agency for Research on Cancer (IARC), a subordinate organization of the World Health Organization (WHO), identified *H. pylori* as a "group 1 (definite carcinogen)"[3]. *H. pylori* infection is identified as the main cause of chronic gastritis, peptic ulcer disease, MALToma[4-6] and gastric adenocarcinoma[7,8].

Many therapeutic regimens have been introduced since the establishment of their pathogenic hypothesis containing bismuth, proton pump inhibitors and one or two antibiotics[9] (mainly tetracyclin, metronidazole, amoxicillin and clarithromycin)[10].

However, considering the evolution of resistant strains of *H. pylori*[11], therapeutic modalities other than antibiotics are necessary and lactobacillus probiotics[12,13] plays a crucial role in this regard[14,15].

Probiotics have the potential to inhibit *H. pylori* due to production of organic acids, peroxides and bactericides[16] as well as competitive inhibition of attachment sites on gastric mucosa. Taking into account that triple or quadruple therapeutic regimens result in 85% to 90% eradication of *H. pylori* makes other therapeutic approaches interesting and necessary in this field.

Declared conflict of interest of all authors: none
Disclosure of funding: no funding received
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Here in, considering the potential role of probiotics in inhibition of *H. pylori* infection and *H. pylori*-induced gastritis, we evaluated the application of honey-derived[17] *Lactobacillus rhamnosus* (*L. rhamnosus*) as a probiotic in the C57BL/6 mice. In this animal study, we tend to compare the results of *H. pylori* infection and *H. pylori*-induced gastritis via stool Ag and Histopathologic exam, respectively between the treatment groups.

METHODS

Animal population

Twenty-four male C57BL/6 mice aged 8-10 weeks were randomly divided into three groups of 8 mice each. Mice were maintained at the animal house under standard conditions (food and water ad libitum, 12:12 hours light/dark cycle, 21±3°C). All experiments involving mice were approved by the Animal Care Committee of Alborz University of Medical Sciences, Karaj, Iran.

H. pylori culture

Helicobacter pylori (Marshall et al.) Goodwin et al. (ATCC® 43504D-5™) was cultured on Brucella agar media containing sheep blood and Trypticase soy agar, in a microaerophilic environment and incubated for 5 to 7 days in 37 degrees of centigrade. After this incubation period, transparent spherical colonies of *H. pylori* appeared which were identified using gram stain and urease, catalase and oxidase tests. The DNA of strain of ATCC® 43504D-5™ was also confirmed with QIAamp DNA Micro Kit (Quiagen).

Animal preparation and induction of *H. pylori* infection

All the mice were fed with 1cc suspension containing 10^8-10^{10} CFU/mL of ATCC43504 strains of *H. pylori* for 3 consecutive days, twice daily via polyethylene gavage tubes. (FIGURE 1) ATCC43504 strains of *H. pylori*.

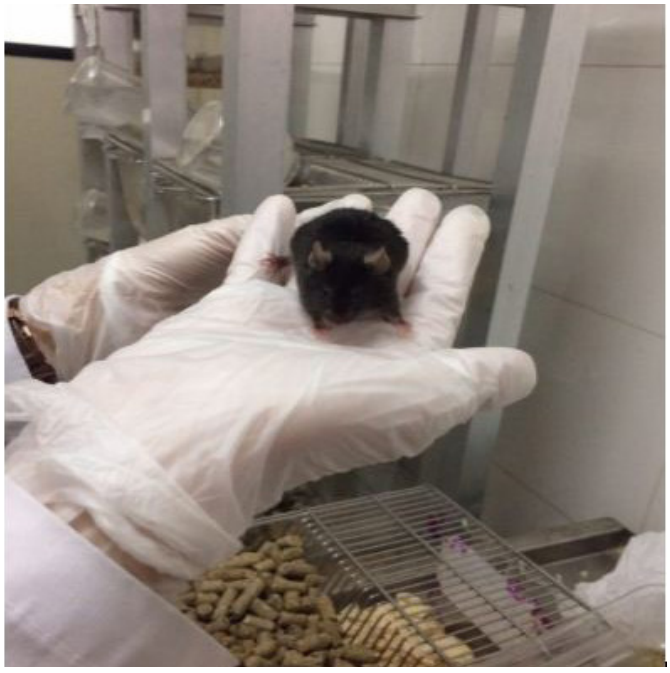

FIGURE 1. Feeding of suspension containing 10^8 CFU/mL of *H. pylori* to the mice via gavage polyethylene tubes.

Confirmation of *H. pylori* infection and gastritis

Infection with *H. pylori* was confirmed with stool Ag (RIA test kits); At the end of 4th week, 30 mg fresh stool was obtained and 1cc of extraction solution was added to the stool tubes. Fifty microliters of stool suspension were added to wells pre-coated with *H. pylori* antibody and incubated for 60 minutes at room temperature. Results were read at 540 nm wavelength and concentrations above 0.505 µg/mL were considered positive for *H. pylori* stool Ag. All mice tested positive at the end of 4th week of ingestion of *H. pylori*.

After treatment all mice killed under anesthetic condition by euthanasia protocole. Mucosal tissue was obtained through a longitudinal incision in the stomach and prepared with hematoxylin-eosin(H&E) and Giemsa staining. Histopathologic study confirmed gastritis in all three.

Preparation of *L. rhamnosus*

L. rhamnosus was derived from honey gathered from mountains of Mazandaran province in northern Iran and cultured in MRS agar media under microaerophilic condition for 48 hours in 37 degrees of centigrade. The colonies were solved in Phosphate-buffered saline (PBS) solution with a dosage of 10^9 CFU/ mL.

Treatment protocols

The mice were subjected to different therapies as stated below;

1: without Bi, Om and *L. rhamnosus* prescription.

2: Daily Bi, Om for the first week and Clarithromycin (Cl) with dosage of 20 mg/kg QD through the second week.

And 3: Daily Bi, Om for the first week plus 1cc of suspension of 10^9 CFU/mL of *L. rhamnosus* QD through the second week.

Evaluation of gastritis and status of *H. pylori* infection after treatment

At the end of the second week of treatment, the stool was tested for Ag and all the remaining seven mice in each group were sacrificed for evaluation of histopathologic changes in the mucosa of stomach. (Stated above in Confirmation of *H. pylori* infection and gastritis section).

RESULTS

The results for stool Ag titer for the seven mice in each treatment group is summarized in TABLE 1.

TABLE 1. *H. pylori* stool Ag titer following treatment.

	#1	#2	#3	#4	#5	#6	#7
Group 1	0.225	0.230	0.525	0.852	1.101	1.011	1.012
Group 2	0.052	0.065	0.045	0.032	0.022	0.00	0.00
Group 3	0.050	0.055	0.065	0.045	0.012	0.00	0.00

Considering 0.505µg/ml as the threshold for a positive stool Ag test, infection persisted in 71.4% of the subjects in the first group following treatment; however, all seven mice were successfully treated in both the clarithromycin and *L. rhamnosus* groups.

Histopathology also revealed the resolution of inflammation in gastric mucosa of all mice following treatment with clarithromycin as well as probiotic *L. rhamnosus*. (FIGURE 2).

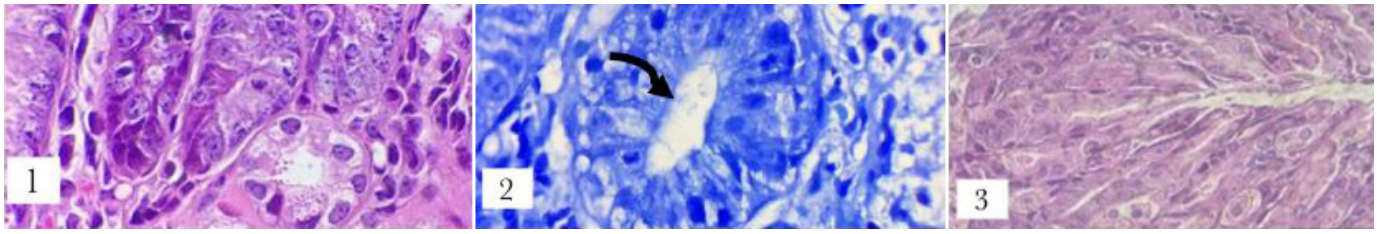

FIGURE 2. Histopathologic slides of gastric mucosa of C57BL/6 mice under * 100 magnification (1: H&E staining of normal gastric mucosa prior to infection, 2: Giemsa staining of infected mouse with black arrow pointing to the *H. pylori* attached to the gastric mucosa via its flagella, 3: H&E staining, confirming the resolution of inflammation following treatment with *L. rhamnosus*.

DISCUSSION

Helicobacter pylori has been the cornerstone of studies addressing gastritis and gastric cancer after its introduction by Marshall and Warren in 1983. Studies on the epidemiology, genetics, pathophysiology and treatment of *H. pylori* has been overwhelming within the last three decades. *H. pylori* is equipped with several virulence factors[18] such as flagella, colonization factors, urease and peroxidase enzymes and vacuolating cytokine A (Vac A)[19] which has made it a very potent agent evading human immune system and resistant to antimicrobials.

Antibiotics has been presented as a potent treatment against *H. pylori* infection causing significant reduction in the rate of gastritis and cancers associated with this infection. Amoxicillin, tetracyclin, metronidazole, clarithromycin and levofloxacin[20] are the main antibiotics utilized in eradication of *H. pylori* infection. However, concerns has arised regarding the use of antibiotics as the resistant strains appeared in the last two decades. Reports of antibiotic resistance vary in different countries. For example, pharmacoepidemiologic studies in Iran, reported resistance to metronidazole, amoxicillin and clarithromycin in 60%, 40%-50% and 30% of the population, respectively.

Beside evolution of resistance, antibiotics sustain several side effects including GI irritation, appearance of infections due to changes in normal flora, influence on the pharmacokinetics of other medications, etc, which has resulted in seeking for alternative therapeutic options.

Probiotics has emerged as novel treatment option in treating several infectious conditions such as bacterial vaginosis, gastrointestinal ulcer and burns. Probiotics may play a role as adjunctive treatment in *H. pylori* infections and possibly in prophylaxis. A wide range of probiotic strains (*L. acidophilus*, *L. johnsonii*, *L. gasseri*, *Bifidobacterium longum* and bioyoghurts) have been studied in *H. pylori* infection and the results are variable[21]. In this study we investigated the effect of sequencial treatment (following adminstration of bismuth and omeprazole) with *L. rhamnosus*, derived from honey, on *H. pylori* infection in C57BL/6 mice.

Previous studies have revealed that *L. rhamnosus* is effective against *H. pylori* infection following antibiotic therapy (as a prophylactic measure to reduce reinfection). This study shows that it is useful as a therapeutic option, and is as effective as antibiotic therapy as the sole treatment to eradicate *H. pylori*.

We hypothesize that the efficacy of *L. rhamnosus* as a therapeutic to eradicate *H. pylori* may rely on bismuth's effect on depolarization of *H. pylori* membrane[22,23] prior to adminstration of *L. rhamnosus* which makes it vulnerable to the bacteriocines released from this probiotic.

Another interesting issue to be mentioned is the derivation of *L. rhamnosus* colonies from honey, which might justify the several studies that present honey as a remedy for *H. pylori* infection[24-29].

A limitation of other studies evaluating the effect of honey on *H. pylori* infection could be the extraction of different bacterial species from different honey types and sources. In the current study, we identified *L. rhamnosus* with genetic sequencing which should be examined with other honey types as well. This animal study warrants a human clinical trial with honey-derived *L. rhamnosus* as a treatment of *H. pylori* infection.

CONCLUSION

Honey-derived *Lactobacillus rhamnosus* is as effective as clarithromycin in eradication of *H. pylori* infection and cure of gastritis, in the C57BL/6 mice.

Authors' contribution

Asgari B: executing tests and acquisition of data. Kermanian F: performing specialised tests and interpretation of data. Derakhshan N: writting and professional editting of manuscript critically. Asna-Ashari M: search literature and data collection. Sadat ZRN: data collection. Yaslianifard S: conception and design writting of text critical revision of the article for important intellectual content. At the end all authors approved the final revision of the manuscript.

Asgari B, Kermanian F, Derakhshan N, Asna-Ashari M, Sadat ZRN, Yaslianifard S. O mel-derivado de *Lactobacillus rhamnosus* alivia a infecção gastro-intestinal induzida pelo Helicobacter pylori-e inflamação gástrica em C57Bl/6 ratos: um estudo imuno-histológico. Arq Gastroenterol. 2018,55(3):279-82.

RESUMO – **Contexto** – O *Helicobacter pylori* (*H. pylori*) foi reconhecido em 1983 por Marechal e Warren como protagonista principal na fisiopatologia de gastrite e úlceras gástricas. Quase metade da população mundial está infectada por *H. pylori*. O regime terapêutico atual contra *H. pylori* inclui o uso de um inibidor da bomba de prótons associada a dois ou mais antibióticos. No entanto, a eficácia deste regime está diminuindo principalmente devido à resistência aos antibióticos e efeitos colaterais de medicamentos. Este fato resultou no interesse público em outras opções terapêuticas e o papel dos probióticos merece atenção especial a este respeito. **Objetivo** – Este estudo visa avaliar a eficácia do mel-derivado do *Lactobacillus rhamnosus* na inflamação gástrica e infecção gastrointestinal *H. pylori*-induzida em camundongos C57Bl/6. **Métodos** – Vinte e quatro camundongos C57Bl/6 foram divididos aleatoriamente em três grupos de oito camundongos cada. Todos os ratos foram alimentados com suspensão de 1cc contendo 5*1010 UFC/mL de cepas ATCC43504 de *H. pylori* por 3 dias consecutivos, duas vezes por dia através de gavagem por tubos de polietileno. No final da 4ª semana, a infecção com *H. pylori* foi confirmada pelo antígeno fecal (ELISA) e após o sacrifício de um rato de cada grupo, o estudo histopatológico confirmou gastrite. Os grupos foram submetidos a diferentes terapias, como indicado, 1: sem prescrição de bismuto (BI), Omeprazol (Om) e *L. rhamnosus*, 2: Bi, Om e claritromicina (CL) e 3: Bi, Om mais 1cc de suspensão de 109 UFC/mL de *L. rhamnosus*. Após 2 semanas, as fezes foram analisadas para o antígeno e os ratos foram sacrificados para a avaliação das alterações histopatológicas. **Resultados** – O tratamento com o grupo *L. rhamnosus* forneceu o título zero de antígeno e foi associado com a inflamação gástrica melhorada em todos os camundongos, similar ao grupo claritromicina. **Conclusão** – O probiótico mel-derivado *L. rhamnosu*s fornece resultados semelhantes ao da claritromicina em termos de melhoria da infecção *H. pylori* e gastrite em C57Bl/6 camundongos modelos, sem os inconvenientes de resistência aos antibióticos.

DESCRITORES – *Helicobacter pylori*. *Lactobacillus rhamnosus*. Úlcera gástrica. Camundongos Endogâmicos C57BL. Mel.

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