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IN-VITRO EVALUATION OF ANTI-*HELICOBACTER PYLORI* ACTIVITY OF COMMERCIALLY AVAILABLE PROBIOTICS

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ABSTRACT: *Helicobacter pylori* is a major etiological agent responsible for several gastric diseases such as gastritis, peptic ulcer disease, and gastric cancer. From the last two decades, standard triple therapy is mostly recommended for *H. pylori* eradication; however, its efficacy is restricted by antibiotic resistance. Hence, the present study aimed at exploration for novel therapeutic agents being warranted to overcome antibiotic-associated limitations in the current *H. pylori* eradication regimens. *H. pylori* DSMZ 10242 was procured and maintained at standard conditions. Anti-microbial assays were performed (in triplicates) to determine the susceptibility of this gastric pathogen to the selected probiotic formulations (Darolac-Z, Pre-Pro, Sporlac, VSL#3, and Yakult) and commonly prescribed antibiotics. The study convincingly reports that Darolac-Z containing *Lactobacillus rhamnosus* and *Saccharomyces boulardii* possess stronger anti-*H. pylori* activity (24.17 mm in 20 h of incubation) in comparison to other probiotics and antibiotics (maximum inhibitory zone observed is 18.4 mm after 48 h of incubation in case of amoxicillin). The probiotic supplementation containing *L. rhamnosus* and *S. boulardii* has a synergistic effect on the inhibition of *H. pylori* growth due to competitive inhibition or production of certain compounds that may possess therapeutic potential as recorded in previous studies. Moreover, in the future, it might be quite interesting to study the role of metabolic by-products of these two strains in the treatment of *H. pylori* induced gastric disorders *in-vivo*.

INTRODUCTION: *Helicobacter pylori*, earlier named as *Campylobacter pylori*, have been perceived as an ancient member of the human microbiota that generally inhabits the human stomach. The colonization of this very pervasive pathogen may prompt a scope of neurotic conditions like atrophic gastritis, chronic gastritis, peptic ulcers and gastric adenocarcinoma [1].

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In 1994, World Health Organization clustered *H. pylori* among group I most potent carcinogens, which has led to the investigation of prophylactic and therapeutic solutions for its eradication [2]. The rate of *H. pylori* infection is on the ascent nowadays; therefore, several clinical investigations have been conducted to find out highly active anti-*H. pylori* therapies.

Till date, the standard treatment for *H. pylori* infection is triple therapy, involving a combination of two antibiotics (clarithromycin and amoxicillin) and one proton pump inhibitor (metronidazole, rabeprazole, esomeprazole, lansoprazole, omeprazole, or pantoprazole) administered for 7-14 days [3].

In recent years, the worldwide accomplishment of antibiotic-based *H. pylori* eradication has declined due to the emergence of antibiotics resistance and has declared to be a general medical issue that imperils significantly the rate of treatment [4]. In addition, antibiotic based eradication is also being linked with several side effects such as abdominal pain, bloating, diarrhea, nausea, and vomiting [5]. Such antibiotic mediated physiological disturbances can be alleviated through the utilization of probiotics, which have convincingly addressed several related etiological conditions in animals and humans [6]. Hence, a search for novel and highly effective probiotic-based anti-*H. pylori* therapy is suggested [4].

Probiotic use is characterized as the real use of live beneficial microbes to obtain a desired outcome by preventing a diseased state or improving general health. Various investigations have affirmed the good impacts of probiotic use in modulating immunity amongst animals and humans. As such, probiotics utilization is picking up prevalence around the world [6]. There is a scarcity of scientific evidence in the literature that supports the mechanism of disease prevention and cures when probiotic-based therapeutic measures are adopted with pharmacological agents. But for sure, existing evidence favor probiotics as the most economical, safer, and widely recommended substitute to triple therapy for reducing *H. pylori* infection in humans [7]. Probiotics follow a variety of immunological and non-immunological modes to eradicate *H. pylori* from gastric epithelium [8]. Among non-immunological mechanisms, pro-biotics strengthen the mucosal barrier (first line of defense) by producing antimicrobial substances such as bacteriocins, hydrogen peroxide, lactic acid, and short-chain fatty acids.

While in immunological mechanisms, probiotics interact with epithelial cells and modulate the secretion of anti-inflammatory cytokines resulting in the reduction of gastric activity and inflammation by inhibiting the secretion of IL-8 [5]. Hence, keeping in view these significant properties of probiotics, further research is required for the exploration of the immense therapeutic potential of probiotic strains. So, in the present study, anti-*H. pylori* activity of commercially accessible five probiotics, namely Darolac-Z, Pre-Pro, Sporlac, VSL#3, and Yakult has been contemplated, and the study evidenced the therapeutic significance of probiotics, designating them as a better alternative to antibiotics for *H. pylori* eradication.

MATERIALS AND METHODS:

Procurement and Maintenance of *H. pylori*: *H. pylori* Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) 10242 was procured from DSMZ, Germany. The culture was maintained in Brain Heart Infusion (BHI) media (HiMedia) supplemented with 5-10% blood at 37 °C for 48 h in a CO_2 incubator maintained at 5% CO_2 level throughout the study as described by DSMZ. The culture used was also maintained as glycerol stock and stored at -20 °C for further utilization [9].

Procurement and Maintenance of Probiotics: A total of 5 commercially available probiotics viz. Darolac-Z, Pre-Pro, Sporlac, VSL#3, and Yakult **Table 1** were purchased, revived, and subcultured in de Man Rogosa Sharpe (MRS - ammonium citrate 2 g/l, beef extract 10 g/l, dextrose 20 g/l, dipotassium phosphate 2 g/l, $MgSO_4$ 0.01 g/l, $MnSO_4$ 0.05 g/l, peptone 10 g/l, sodium acetate 5 g/l, tween-80 1 ml/l, yeast extract 5 g/l, pH 6.5) broth comprising 1-1.5 billion spores each and maintained at 37 °C for 18-24 h [10].

TABLE 1: COMPOSITION OF COMMERCIALLY AVAILABLE PROBIOTIC FORMULATIONS USED IN THE PRESENT STUDY

S. no.	Probiotic Formulation	Composition	Spore Count
1	Darolac Z (2g sachet)	*Lactobacillus rhamnosus*, *Sacharomycesboulardii*	1×10^9
2	Pre-Pro (1g sachet)	*Bacillus mesentericus*, *Clostridium butyricum*, *L. acidophilus*, *Streptococcus faecalis*	$\sim 1 \times 10^9$
3	Sporlac (1g sachet)	Lactic acid Bacillus (earlier known as *L. sporogenes*)	150×10^6
4	VSL#3 (1 capsule)	*L. acidophilus*, *L. delbrueckii subsp. bulgaricus*, *L. paracasei*, *L. plantarum*, *Bifidobacterium breve*, *B. infantis*, *B. longum*, *Streptococcus thermophilus*	112.5×10^9
5	Yakult (65ml/package)	*L. casei*	6.5×10^9

Preparation of Antimicrobial Extracts of Probiotic Formulations: 1 ml aliquot of each probiotic broth culture was collected and heat treatment was given in a boiling water bath at 80 °C for 10 min. After cooling for 5 min at room temperature, samples were centrifuged at 12,000 rpm for 10 min and cell free supernatant (CFS) was collected in a fresh vial. Further, probiotic activity was assayed using agar well diffusion method [9].

Anti-microbial Assays of Probiotic Formulations: To assay anti-microbial activity of probiotics, well diffusion assay was performed in triplicates as per Kaur and co-workers with some modifications [11]. BHI agar (2.5%, *w/v*) bottom layer was overlaid with BHI soft agar (0.75%, *w/v*) media comprising 5-10% fresh blood and pre-inoculated with *H. pylori* (0.1%, *v/v*) culture. Wells of 8 mm diameter were punched using sterile cork borer and 50 µl of the extracts were added to each well and finally, the plates were incubated at 37 °C for 24-48 h in CO_2 incubator. Plates were observed for inhibition zones, and the assay was considered positive if the diameter of inhibition zones appeared around the wells exceeded 1 mm in size.

Antibiotic Susceptibility of *H. pylori*: Antibiotic susceptibility of gastric pathogen *H. pylori* was tested using HiMedia Antibiotic OCTA discs by performing activity assay. BHI agar (2.5%, *w/v*) was poured in petriplate and kept for solidification. It was further overlaid with BHI soft agar (0.75%, *w/v*) pre-seeded with *H. pylori* (0.1%, *v/v*) overnight grown culture. Now, antibiotic discs were placed aseptically on the solidified agar plates and incubated at 37 °C for 24-48 h in CO_2 incubator and zones of inhibition were measured [11].

Statistical Analysis: Data of all experiments were performed in three replicates and expressed as mean values. The statistical analysis using Microsoft Excel (MS Office 10) was carried out to calculate the standard deviation.

RESULTS AND DISCUSSION: For the past 15 years, a triple regimen has been considered as the standard therapy against *H. pylori* infection. However, the increase in the prevalence of antibiotic resistance has decreased the efficacy of such therapies to unacceptably low levels in most parts of the world, resulting in the necessity of studying other possible therapies in order to eradicate the pathogen at the global scale [12]. Hereby, probiotics are being proposed as GRAS, economically viable, and large-scale therapeutic alternatives for *H. pylori* eradication **Table 2**.

TABLE 2: *IN-VITRO* STUDIES DEPICTING ANTIMICROBIAL ACTIVITY OF VARIOUS PROBIOTIC FORMULATIONS AGAINST *H. PYLORI*

Probiotic Strain Involved	Zone of Inhibition (mm)		Incubation time	References
L. sakei *L. plantarum* *L. rhamnosus* *L. brevis*	12, 15, 12, 15		72 h	Sun*et al*., 2018[15]
L. casei Shirota *L. fermentum* UCO-979C *L. johnsonii* La1 *L. rhamnosus*	4.0, 6.0, 3.0, 3.0		72 h	García *et al*., 2017[16]
L. casei (LOCK 908) *L. paracasei* (LOCK 919) *L. plantarum* (LOCK 862) *L. rhamnosus* (LOCK 900)	4.8, 3.6, 2.3, 5.21		5 days	Wiese *et* al., 2015[17]
L. acidophilus (LY5) *L. bulgaricus* (LY1) *L. paracasei* (IF22)	10.0, 9.7, 10.0		48 h	Lin *et al.*, 2011[18]
B. subtilis 3	16.0		48 h	Qureshi*et al*., 2019[7]
Darolac Z	*L. rhamnosus S. boulardii*	24.17	20 h	Present study
Pre-Pro	*B. mesentericus C. butyricum* *L. acidophilus S. faecalis*	18.83	20 h	Present study
Sporlac	Lactic acid Bacillus (*L. sporogenes*)	19.17	20 h	Present study
VSL#3	*L. acidophilus L. delbrueckii subsp. Bulgaricus L. paracasei* *L. plantarum B. Breve B. infantis* *B. longum S. thermophilus*	20.83	20 h	Present study
Yakult	*L. casei*	21.0	20 h	Present study

In the present study, the five selected probiotics were revived and maintained in MRS medium under specified conditions **Suppl Fig. 1.** The antimicrobial activity of cell-free extracts of all five probiotics (Darolac-Z, Pre-Pro, Sporlac, VSL#3, and Yakult) was determined against *H. pylori* DSMZ 10242 by agar well diffusion assay. All the antibiotics and probiotic formulations used in the study showed inhibitory activity against *H. pylori*, as evidenced from **Table 3** and **Fig. 1**.

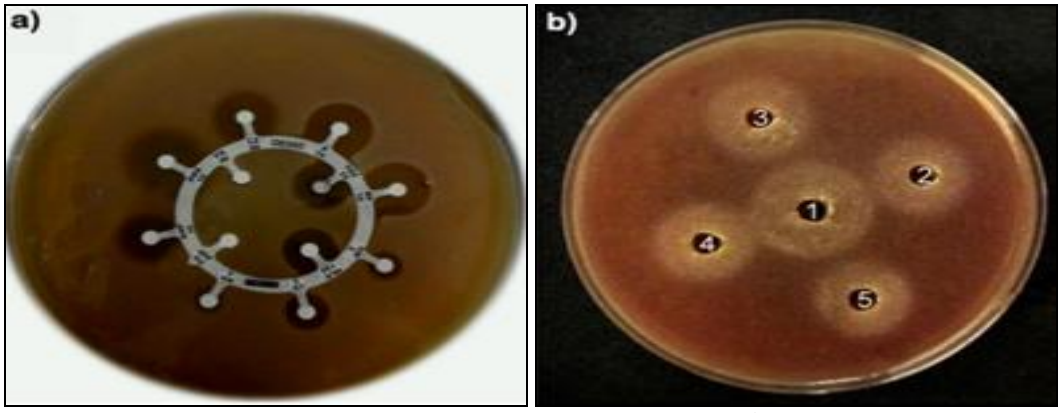

FIG. 1: ASSAY PLATES DEPICTING ANTI-*H. PYLORI* ACTIVITY OF A) PURE ANTIBIOTICS AND B) FIVE COMMERCIALLY AVAILABLE PROBIOTIC FORMULATIONS WHERE, WELL 1: DAROLAC-Z, 2: PRE-PRO, 3: SPORLAC, 4: VSL#3 AND 5: YAKULT

According to the data reported in antibiogram studies determined using OCTA-discs (Himedia, India), *H. pylori* were found to be resistant to fosfomycin, norfloxacin, and vancomycin. However, the highest inhibitory activity was observed in the case of amoxicillin (18.4 mm) and lowest in the case of ciprofloxacin (3.90 mm) after 48 h of incubation. Interestingly in the case of probiotic formulations, *H. pylori* inhibition was recorded at 20th hour of incubation, and the maximum inhibition was obtained using Darolac-Z with an inhibition zone of 24.17 mm, followed by Yakult (21 mm), VSL#3 (20.83 mm), and Sporlac (19.17 mm). At the same time, minimum inhibition was recorded in the case of Pre-Pro with an 18.83 mm inhibitory zone around the well.

In the present study, anti- *H. pylori* activity of five most recommended probiotic formulations for gastritis, namely Darolac-Z, Pre-Pro, Sporlac, VSL#3, and Yakult was comparatively investigated. Among them, Darolac-Z formulation comprising probiotics *L. rhamnosus* and *S. Boulardii* showed maximum activity against *H. pylori* (with an inhibitory zone of 24.17 mm) obtained within 20th hour of incubation as evidenced from *in vitro* assay plates.

Whereas, in the case of antibiotics, maximum anti-*H. pylori* activity was observed around amoxicillin discs with an 18.4 mm zone of inhibition after 48 h of incubation. These results provide conclusive evidence to figure out the higher efficacy and remarkable inhibitory potential of probiotics against *H. pylori* proliferation in **Table 3**.

TABLE 3: COMPARATIVE ACCOUNT OF ANTI-*H. PYLORI* ACTIVITY OF ANTIBIOTICS *vs.* COMMERCIALLY AVAILABLE PROBIOTIC FORMULATIONS

S. no.		Zone of Inhibition* (mm)
Pure Antibiotics		
1	Amoxycillin (AMX)	**18.4 ± 0.05**
2	Ampicillin (AMP)	13.3 ± 0.14
3	Ciprofloxacin (CIP)	3.90 ± 0.03
4	Erythromycin (E)	17.2 ± 0.04
5	Fosfomycin (FO)	No Zone
6	Gentamycin (HLG)	14.70 ± 0.06
7	Levofloxacin (LE)	8.60 ± 0.03
8	Linezolid (LZ)	15.60 ± 0.05
9	Norfloxacin (NX)	No Zone
10	Pristhiomycin (RP)	17.4 ± 0.04
11	Tigecycline (TGC)	15.6 ± 0.04
12	Vancomycin (VA)	No Zone
Probiotic Formulations		
1	Darolac-Z	**24.17 ± 0.89**
2	Pre-Pro	18.83 ± 1.09
3	Sporlac	19.17 ± 0.6
4	VSL#3	20.83 ±1.17
5	Yakult	21.0 ± 1.26

*The data are presented as mean ± SD (N=3 experiments)

All the tested probiotic formulations gave better results than individual antibiotics in terms of inhibition of *H. pylori* growth during *in vitro* assessment. Interestingly, *L. rhamnosus* has been documented as the world's best probiotic bacterium

with distinct characteristics of an 'ideal' probiotic like being resistant to acid and bile secretions, adherence, antimicrobial production, immune stimulation, and other health benefits [13]. Earlier, studies also stated that probiotic *L. rhamnosus* supplementation has a positive impact on *H. pylori* therapy-related side effects and treatment tolerability [14].

Survival of *H. pylori* in Stomach: *H. pylori* has a unique life-supporting system for its survival in extreme acidic conditions of the stomach. It has an acid gated membrane channel that regulates the production of alkali by the bacterium itself to combat the acidic environment for its survival and growth [19]. Basically, *H. pylori*, upon activation in the stomach, express two proteins *i.e.*, Ure I (a member of amidoporin family of proteins) and an adhesion protein Bab A.

Ure I regulates the passage of urea into the cytoplasm of the bacteria *via* channels across the cell membrane for the production of ample amount of ammonia in order to neutralize periplasm that helps the bacterium to resist acidic conditions in the stomach [19]. Whereas, the specific binding affinity of Bab A to naturally expressed antigens on healthy gastric epithelium Lewis^b helps in maintaining direct contact with the epithelium as well as the recoil from of the risk for being cleared from the stomach [20].

Role of *Lactobacilli* in *H. pylori* Eradication Therapy: Earlier, a study was conducted to investigate the effects of pH, organic acids such as lactic acid, acetic acid, and various probiotic strains such as *B. bifidus*, *L. acidophilus*, *L. bulgaricus*, *L. casei*, and *Pediococcus pentosaceus* on *H. pylori* growth [21]. Organic acids showed *H. pylori* growth retardation in a concentration-dependent manner. And among all the tested probiotic strains, *L. acidophilus* and *L. casei* subsp. *rhamnosus* were found to the best in the context of *in vitro* inhibition of *H. pylori* growth [21]. Further, co-fermentation and an *in vivo* study revealed the antimicrobial activity of three *Lactobacillus* strains (*L. gasseri*, LG21, and *L. salivarius* WB1004) against *H. pylori*. LG21 and *L. salivarius* WB1004 upon oral administration eradicated *H. pylori* while the levels of anti-*H. pylori* IgG of LG21-administered mice was found to be the lowest [22]. In evidence of this, Ushiyama and the team stated LG21 probiotic as an effective probiotic in case of clarithromycin-resistant *H. pylori* infection [23]. Whereas, in 2008, a strain-dependent study was conducted to investigate the effect of different strains of *L. salivarius* isolated from distinct environmental niche or geographic locations on the *H. pylori* growth. The study stated that irrespective of sites and geographical location, anti-*H. pylori* activity of *L. salivarius* is consistent. And inhibition necessitates the presence of live cells, rather than acid production or by some protein secretion [24].

Later on, several other probiotic strains such as *L. acidophilus*, *L. rhamnosus*, *B. longum*, and *B. bifidum* were investigated for inhibiting the growth of enteric pathogen *Enterococcus faecalis* and *Candida albicans in-vitro* [25]. In addition to anti-*H. pylori* activity, *L. rhamnosus* GG (ATCC 53103) also produces growth inhibitory substances against *Streptococcus sobrinus* that reduces the risk of dental caries [26]. Later on, Bohora and Kokate also acknowledged LGG as a potential endodontic intracanal medicament in 2017 [25].

Besides being anti-bacterial in nature, LGG has been well documented in treating protozoan infections such as Giardiasis, where *Giardia* trophozoites adhere to the epithelial surface of the small intestine, causing diarrhea, malnutrition, and growth retardation. Probiotic LGG has the potential to displace the trophozoites through competitive exclusion, thereby showing a better ability to adhere and colonize in murine enterocytes [27].

Role of *S. boulardii* in *H. pylori* Eradication Therapy: Interestingly, *S. boulardii* has been reported to reduce the colonization of *H. pylori* in the human stomach and have a magnificent effect on the prevention and treatment of diarrhea during *H. pylori* eradication [28-29]. A study by Szajewska and co-workers documented a convincing meta-analysis report stating the additive effect of *S. boulardii* with triple therapy in reducing the risk of adverse side effects of overall *H. pylori* therapy, specifically diarrhea and epigastric discomfort [30]. In addition, the yeast significantly enhanced the treatment efficacy by mucosal-anti-inflammatory signaling property that suppresses the synthesis of inflammatory cytokines [29].

Proposed Mechanisms of *H. pylori* Inhibition by Lactic Acid Bacteria (LAB): Pathways of *H. pylori* inhibition by LAB are manually documented in several studies and mainly comprised of the followings:

Inhibition of Urease Activity: *H. pylori* urease enzyme is the life-supporting system of this gastric pathogen that decomposes urea into NH_3 and CO_2, thus raising pH of the microenvironment around *H. pylori* colonies that enables it to survive and proliferate in the highly acidic gastric environment. A study by Sun *et al.*, reported that *L. brevis* and *L. rhamnosus* both inhibit urease activity of *H. pylori* that plays an important role in its eradication[15].

Competitive Exclusion Through Production of Chemo-active Antimicrobials: Interaction of the *H. pylori* to gastric epithelial cells is magnificently restricted by the production of probiotic associated secretory components such as organic acids, short-chain fatty acids, and other chemoactive agents like antibiotics and bacteriocins. Probiotic *Bacillus subtilis* 3 strain was shown to inhibit *H. pylori* due to the secretion of antibiotic substances like amicoumacin A that also possesses anti-inflammatory and anti-ulcer properties as tested in rat models [7].

Besides this, the binding affinity of *H. pylori* to specific glycolipid receptors asialo-GMI and sulfatide has been hampered by *L. reuteri* [31]. An *in-vivo* study confirms the reduced *H. pylori* infection upon prior colonization by probiotics [32]. For competitive exclusion, probiotic strains must share glycolipid specificity as of *H. pylori* [3]. Recently, Westerik and co-workers reported a harmless novel relationship of probiotic *L. rhamnosus* GG (LGG) with *H. pylori* that reduces gastric pathology as a result of competition for receptor binding sites on the gastric epithelium. *L. rhamnosus* yoba 2012 (LRY), the generic variant of LGG, inhibits *H. pylori* through competitive exclusion and production of antimicrobial agents such as lactic acid.

The authors convincingly proposed that supplementation of LRY in diet could establish a subsequent *H. pylori* eradication therapy treatment [33]. Further, Chen and co-workers investigated the therapeutic potential of *L. rhamnosus* JB3 and two flavonoids (baicalin and baicalein) against *H. pylori* [34]. A synergistic effect of *L. rhamnosus* JB3 and baicalein was reported in the study as evidenced by stronger anti- *H. pylori* activity of combination of 1×10^6 colony-forming units [CFUs]/ml of probiotic with 125 μM of baicalein. Synergistic antimicrobial effect was exhibited through interference with adhesion and invasion ability of *H. pylori* to cells by suppressing *vacA* gene expression and suppression of IL-8 expression.

Attenuation of Host Apoptotic and Inflammatory Responses and Angiogenesis in Infected Tissue: In intestinal epithelial cell models, studies revealed the anti-*H. pylori* property of *L. rhamnosus* GG to avert cytokine-induced apoptosis by inhibiting the activation of proapoptotic p38/mitogen-activated protein kinase by tumor necrosis factor (TNF) [35]. *L. rhamnosus* also alters the release of *H. pylori* induced IL-8 and TNF-α thus attenuating the levels of gastrin-17 that helps in providing a friendly microenvironment to *H. pylori* [36].

Epithelial cells adopt different criteria to recognize bacterial DNA; in the case of pathogenic bacteria, phosphorylation of extra-cellular signal-induced kinase pathway has been activated while for probiotics stimulation of nuclear factor-κB pathway in response to TNF-α has been reported [3, 37].

In addition to competitive exclusion, *L. rham-nosus* yoba 2012 (LRY) also inhibits *H. pylori* through elevating the expression of COX-2 protein, further inducing the expression of vascular endothelial growth factor (VEGF) that finally stimulate angiogenesis in the *H. pylori*-induced peptic ulcers [33].

Restoration of Mucosal Barrier: Upon infection and proliferation in the human gastric epithelium, *H. pylori* suppress the gene expression of MUCI and MUC5A, resulting in reduced secretion of mucus, disrupting the mucosal permeability of gastric epithelium [7]. Intake of probiotics such as *L. plantarum* and *L. rhamnosus* helps in strengthening of the mucosal barrier in the stomach by stimulating mucin production due to increased expression of MUC2 and MUC3 genes and consequently restoring mucosal permeability of gastric mucosa - the first line of gastric defense [5].

CONCLUSION: Present study provides experimental evidence supporting anti-*H. pylori* activity of various commercially available pro-biotics. Probiotic formulation Darolac-Z showed maximum *in-vitro* inhibition of gastrointestinal carcinogenic pathogen *H. pylori*. Maximum inhibitory activity was reported within 20 h of incubation, which is the shortest among recognized antibacterials reported time till date. Consistent inhibitory results were obtained till 48 h of incubation. However, in the case of pure antibiotics, a clear zone of inhibition was observed after 48 h of incubation, which indicates better performance of probiotics with respect to antibiotics. However, the involvement of probiotic organisms in *H. pylori* eradication, their synergistic action with antibiotics, and the detailed mechanism of growth inhibition and significant immuno-modulation role of metabolic end-products in the eradication of *H. pylori* need further research.

Further, *in-vivo* trials are recommended to prove the efficacy of probiotics in clinically proven *H. pylori*-infected patients. The results of the present study are quite exhilarating as over the past decade emergence of 'superbugs' has augmented spectacularly which implies a growing need to introduce an innovative therapeutic strategy that relies on the secretion of antimicrobial molecules and significant metabolic by-products involved in eradication therapy.

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CONFLICTS OF INTEREST: The authors declare that they have no conflict of interest.

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