


Bovine lactoferrin enhances the efficacy of levofloxacin-based triple therapy as first-line treatment of *Helicobacter pylori* infection: an *in vitro* and *in vivo* study

Antonio Francesco Ciccaglione^{1†}, Mara Di Giulio^{2†}, Silvia Di Lodovico², Emanuela Di Campli², Luigina Cellini¹ ^{2*} and Leonardo Marzio¹

¹Digestive Sciences Unit, 'G. d'Annunzio' University, Pescara Civic Hospital, Via Fonte Romana 8, 65124, Pescara, Italy; ²Department of Pharmacy, 'G. d'Annunzio' University, Via dei Vestini, 66013, Chieti, Italy

*Corresponding author. Tel: +39-0871-3554560; Fax: +39-0871-3554562; E-mail: l.cellini@unich.it  orcid.org/0000-0003-4068-9124

†These authors contributed equally to the work.

Received 5 July 2018; returned 17 September 2018; revised 24 October 2018; accepted 12 November 2018

Objectives: To evaluate the *in vitro* antimicrobial/antivirulence action of bovine lactoferrin and its ability to synergize with levofloxacin against resistant *Helicobacter pylori* strains and to analyse the effect of levofloxacin, amoxicillin and esomeprazole with and without bovine lactoferrin as the first-line treatment for *H. pylori* infection.

Methods: The bovine lactoferrin antimicrobial/antivirulence effect was analysed *in vitro* by MIC/MBC determination and twitching motility against six clinical *H. pylori* strains and a reference strain. The synergism was evaluated using the checkerboard assay. The prospective therapeutic trial was carried out on two separate patient groups, one treated with esomeprazole/amoxicillin/levofloxacin and the other with esomeprazole/amoxicillin/levofloxacin/bovine lactoferrin. Treatment outcome was determined with the [¹³C]urea breath test.

Results: *In vitro*, bovine lactoferrin inhibited the growth of 50% of strains at 10 mg/mL and expressed 50% bactericidal effect at 40 mg/mL. The combination of levofloxacin and bovine lactoferrin displayed a synergistic effect for all strains, with the best MIC reduction of 16- and 32-fold for levofloxacin and bovine lactoferrin, respectively. Bovine lactoferrin at one-fourth MIC reduced microbial motility significantly for all strains studied. In the *in vivo* study, 6 of 24 patients recruited had treatment failure recorded with esomeprazole/amoxicillin/levofloxacin (75% success, 95% CI 57.68%–92.32%), and in the group with esomeprazole/amoxicillin/levofloxacin/bovine lactoferrin, 2 out of 53 patients recruited had failure recorded (96.07% success, 95% CI 90.62%–101.38%).

Conclusions: Bovine lactoferrin can be considered a novel potentiator for restoring susceptibility in resistant *H. pylori* strains. Bovine lactoferrin added to a triple therapy in first-line treatment potentiates the therapeutic effect.

Introduction

Helicobacter pylori is involved in the development of chronic gastritis and peptic ulcer disease and has been linked to the pathogenesis of gastric lymphoma and gastric cancer; hence it is recommended that this infection should be cured whenever it is diagnosed.^{1–3} Clarithromycin, amoxicillin, metronidazole, tinidazole, tetracycline, rifabutin, ampicillin and fluoroquinolones have been used to treat *H. pylori* infection.^{4,5} This bacterial infection, however, has been shown to be challenging to cure. In fact, *H. pylori* may be resistant in various degrees to one or more of the above-mentioned antibiotics, even in subjects never treated specifically for the infection, and antibiotic resistance may be a key factor in treatment failure.⁶ This alarming phenomenon strongly

supports the need to find novel strategies, such as the inclusion of an adjuvant aimed at enhancing the effectiveness of drugs commonly used in this specific therapy.⁷

Classical triple therapies with proton pump inhibitors, clarithromycin and amoxicillin or metronidazole are the mainstay of current treatment; the increasing resistance to clarithromycin, however, has reduced their effectiveness, with an eradication rate of <80% of treated cases.^{8,9} A recent consensus report suggests that a proton pump inhibitor/clarithromycin-containing triple therapy without prior susceptibility testing should be abandoned in those areas where the clarithromycin resistance rate is >15%.¹⁰

Resistance rates vary in different geographical areas and therefore the selection of therapeutic regimens needs to be adjusted

according to the local resistance pattern, if this is known.^{11–15} In the region of Abruzzo, the latest data on resistance of *H. pylori*, isolated from already-treated (once or several times) and never-treated patients, to amoxicillin, clarithromycin, metronidazole, levofloxacin, tetracycline and rifabutin are as follows: 1.02%, 72.44%, 34.69%, 42.85%, 2.63% and 1.20%, respectively.¹⁶ Consequently, the geographical prevalence of the *H. pylori* resistance profile should be the basis for the selection of first-line eradication therapy aimed at avoiding primary failure.¹⁷

As an alternative to clarithromycin, many studies have examined levofloxacin and proton pump inhibitors as a first-line therapy for eradication of *H. pylori* infection.¹⁸ However, the eradication rates achieved with first-line levofloxacin-based treatments are not uniform and contrasting results have been reported.^{19–21} In regions of high clarithromycin resistance, bismuth-containing quadruple therapies are recommended for first-line therapy since bismuth may help to overcome the antibiotic resistance to clarithromycin when present.^{22,23} Bismuth salts, however, are not available in all countries; it is therefore useful to search for a valid substitute that may increase the effectiveness of a triple therapy regardless of the presence of *H. pylori* resistance to one or more antibiotics.

Recent data have called attention to the potential role of fermented milk and related whey proteins, such as bovine lactoferrin, as potential candidates for complementary therapy in settings of high antibiotic resistance or treatment failure.²⁴

Bovine lactoferrin is a glycoprotein with multiple antimicrobial, antiviral and antifungal properties and it is widely distributed in mucosal secretions, such as saliva, tears and seminal fluid.²⁵ As Ellison *et al.*²⁶ reported, the antimicrobial activity of bovine lactoferrin is due to the sequestering of iron, which is essential for microorganism growth, and also to a direct action on the outer membrane of Gram-negative bacteria that interferes with flagellar motility. Bovine lactoferrin has been shown to have antibacterial activity against *H. pylori* *in vitro*²⁷ and *in vivo*.²⁸ Clinical studies have demonstrated that when bovine lactoferrin is used as a single drug in *H. pylori*-positive patients it is able to reduce the production of urea, suggesting a capacity to suppress but not eliminate *H. pylori* colonization.^{29,30}

However, it has not been clarified whether bovine lactoferrin has a direct bactericidal effect on *H. pylori* or whether it potentiates the efficacy of a given antibiotic also in the presence of specific resistance.

The aim of this study was 2-fold: firstly to analyse the *in vitro* antimicrobial/antivirulence action of bovine lactoferrin alone and combined with levofloxacin against *H. pylori* clinical isolates previously shown to be resistant or MDR; and secondly to evaluate whether the inclusion of bovine lactoferrin in a triple therapy containing levofloxacin, amoxicillin and a proton pump inhibitor at full dosage for first-line treatment of *H. pylori* infection could increase the eradication rate in a geographical area where *H. pylori* has been shown to be resistant to levofloxacin in >40% of tested strains.

Materials and methods

Effect of bovine lactoferrin on clinical *H. pylori* strains *in vitro*

Characterization of antimicrobial susceptibility and virulence factors

Six *H. pylori* clinical strains were chosen for experiments. The reference *H. pylori* ATCC 43629 strain was included as a control. All strains studied

were tested for their susceptibility profile to nine antibiotics commonly used in therapy (clarithromycin, metronidazole, ciprofloxacin, levofloxacin, moxifloxacin, tetracycline, amoxicillin, ampicillin and rifabutin) and for the main virulence factors.¹⁶

Strains were recovered from -80°C and were cultured on non-selective medium containing Columbia agar base (Oxoid) with 10% (v/v) laked horse blood plus 1% (v/v) IsoVitalax (BBL, Microbiology System, Milan, Italy) and incubated in a micro-aerobic environment at 37°C for 3–5 days (GasPak, Oxoid). The bacterial suspensions were prepared in Brucella Broth (BB) (Biolife Italiana, Milan, Italy) plus 2% FCS (Biolife), adjusted to an OD_{600} of 0.2, corresponding to $\sim 1.8 \times 10^7$ cfu/mL, and used for the experiments.

For virulence factor genotyping, the genomic DNA was extracted with the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA was stored at -20°C until use. PCRs were performed in a 2700 Thermocycler (PE Applied Biosystems) for the analysis of *cagA*, *iceA1* and *vacA s/m/i* status according to previous studies by our group.^{31,32} The PCR was prepared in a total volume of 25 μL containing 2.5 μL of $10 \times$ PCR buffer, 1.5 mM MgCl_2 , 200 mM (each) deoxynucleotide triphosphates (dNTPs), 2 U of AmpliTaq DNA polymerase, 20 mM each primer (Primm, Milan, Italy) and 50 ng of *H. pylori* DNA. For the analysis of the *cagA* region, primers D008 and R008, which yielded a fragment of 298 bp, were used. For the analysis of the *iceA* region, primers *iceA1-F* and *iceA1-R*, which yielded a fragment of 600 bp, were used. For the analysis of the *vacA/s* region, primers VA1-F and VA1-R, which yielded a fragment of 259 bp for the s1 variant and a fragment of 286 bp for the s2 variant, were used. For the analysis of the *vacA/m* region, primers VAG-F and VAG-R, which yielded a fragment of 567 bp for the m1 variant and a fragment of 642 bp for the m2 variant, were used. For the analysis of the *vacA/i* region, primers VAC-F1, CR1 and CR2, which yielded a fragment of 426 bp for the i1 variant and a fragment of 432 bp for the i2 variant, were used.³²

The PCR products were examined by electrophoresis in 2% (w/v) agarose gel at 100 V for 30 min. Gels were stained with ethidium bromide and photographed.

Determination of MIC and MBC of bovine lactoferrin

The *in vitro* antimicrobial activity of bovine lactoferrin (Sigma-Aldrich) was assessed by the broth microdilution method according to CLSI guidelines.³³ Bovine lactoferrin was diluted to obtain a concentration from 2.5 to 320 mg/mL. MIC values were measured by determining the lowest concentration of bovine lactoferrin needed to inhibit the visible growth of the microorganisms. The MBC was determined as the lowest concentration of bovine lactoferrin that gave complete inhibition of colony formation on plates. Each determination was performed in triplicate.

Chequerboard titration analysis

To understand the *in vitro* effect/synergism of bovine lactoferrin and levofloxacin, the chequerboard titration method was applied to assess the activity of the combination of these substances.³⁴ Test tubes containing a sub-MIC concentration of bovine lactoferrin from 0.31 to 20 mg/mL and sub-MIC concentration of levofloxacin from 0.03 to 1 mg/L, respectively, in PBS were prepared and put in 96-well plates for the chequerboard configuration. The overnight bacterial inocula were prepared in BB plus 2% FCS, refreshed in the same medium and adjusted to OD_{600} 0.12 ($\sim 5 \times 10^6$ cfu/mL) for the experiments. The plates were incubated for 3 days at 37°C under micro-aerophilic conditions. After incubation, reduction of the OD_{600} value was evaluated with respect to the controls and cfu/mL values were determined. The chequerboard test was used as the basis to calculate a fractional inhibitory concentration (FIC) index³⁵ according to the following formulas: $\text{FIC A} = \text{MIC A} + \text{B}/\text{MIC A}$; $\text{FIC B} = \text{MIC B} + \text{A}/\text{MIC B}$; and $\text{FIC index} = \text{FIC A} + \text{FIC B}$. The MIC A + B value is the MIC of compound A in the presence of compound B, and vice versa for MIC B + A. FIC index values were interpreted as Odds³⁶ synergy (index ≤ 0.5), antagonism (> 4.0) and no

Table 1. Antimicrobial susceptibility panel and virulence markers of *H. pylori* clinical strains

| Strain | Antibiotic | | | | | | | | | Genotype | | |
|------------|------------|-----|-----|-----|-----|-----|-----|-----|-----|-------------|--------------|-------------|
| | CLR | MTZ | LVX | MXF | CIP | RFB | TET | AMP | AMX | <i>cagA</i> | <i>iceA1</i> | <i>vacA</i> |
| 9F/13 | S | S | S | S | S | S | S | S | S | + | + | s1m1i1 |
| 10A/13 | R | R | R | R | R | S | S | S | S | + | – | s1m2i1 |
| 2A/12 | R | R | R | R | R | S | S | S | S | + | + | s1m1i1 |
| 10A/11 | R | S | R | R | R | S | S | S | S | + | – | s1m1i1 |
| 11F/11 | R | S | R | R | R | S | S | S | S | + | – | s1m2i2 |
| 3A/13 | R | S | R | R | R | S | S | S | S | + | + | s1m2i2 |
| ATCC 43629 | S | S | S | S | S | S | S | S | S | + | – | s1m1i1 |

CLR, clarithromycin; MTZ, metronidazole; LVX, levofloxacin; MXF, moxifloxacin; CIP, ciprofloxacin; RFB, rifabutin; TET, tetracycline; AMP, ampicillin; AMX, amoxicillin; S, susceptible; R, resistant.

interaction (>0.5–4.0). Each determination was performed in triplicate. For the control, bovine lactoferrin and levofloxacin were also assayed alone.

Motility assay

Motility of *H. pylori* strains was assayed using semi-solid medium consisting of BB, sterile 10% FCS, sterile 10% horse blood and 0.4% bacteriological agar. Ten microlitres was inoculated on the surface of the agar with a sterile tip. Plates were incubated at 37°C under micro-aerophilic conditions. Diameters of the spreading *H. pylori* cells were measured after 7–10 days.^{37,38} The statistical significance of differences between diameters of untreated and treated samples was evaluated using Student's *t*-test. Probability levels of <0.05 were considered statistically significant.

All data were obtained from three independent experiments performed at least in triplicate.

Therapeutic trial of bovine lactoferrin in conjunction with levofloxacin, amoxicillin and a proton pump inhibitor

This prospective study was performed in two separate groups of patients in two pilot studies that were carried out simultaneously. One group was treated with esomeprazole, amoxicillin and levofloxacin (Group A) and the other group with esomeprazole, amoxicillin, levofloxacin and bovine lactoferrin (Group B).

For this study, 50 subjects were required for each pilot study. An effective therapy was defined as a PP cure rate of ≥90% and rates of ≤80% were prospectively deemed unacceptable. Initially the plan was to evaluate 30 patients, and the study would have been stopped at any time in each group during recruitment if ≥6 patients experienced failure [urea breath test (UBT) positive], since it became clear that a cure rate of at least 80% would be impossible to achieve. On the other hand, if at the end of recruitment of the 30 patients a cure rate >93% was achieved, the study was considered concluded and no further recruitment was necessary. Otherwise, the study would continue until 50 patients had completed the study.³⁹

Success was assessed by the [¹³C]UBT 2 months after the end of treatment.

Patient selection

From January 2015 to December 2016, of the 92 consecutive UBT *H. pylori*-positive patients never treated for the infection, 77 were included in the study. The UBT was performed with citric acid and 75 mg of [¹³C]urea within 10 days prior to the start of the study.

All eligible patients agreed to participate in the study. Fifteen patients were excluded according to the following criteria: age <18 or >80 years; treatment with proton pump inhibitors (omeprazole, lansoprazole, pantoprazole, rabeprazole, esomeprazole), H2 blockers (ranitidine, nizatidine, cimetidine, famotidine, roxatidine) and/or antibiotics during the 4 weeks before the study; gastrointestinal malignancy; severe concomitant diseases; and previous gastric surgery. All patients enrolled in the study were affected by chronic dyspepsia without alarm symptoms. Only patients >55 years underwent an upper endoscopy before admission to the study. Patients with active gastric and/or duodenal ulcer or gastric neoplasia were excluded.³⁹

Patients were randomly assigned to each treatment using a computer-generated list.

Ethics

Enrolled patients gave informed consent for the study, which was approved by the Scientific Committee for Human Research of the Department of Medical Sciences of 'G. d'Annunzio' University.

Study protocol

Patients included in Group A were treated with esomeprazole (40 mg twice daily), amoxicillin (1 g twice daily) and levofloxacin (500 mg twice daily) and patients included in Group B were treated with the same drugs and dosage as Group A with the addition of bovine lactoferrin (200+100 mg/day). Esomeprazole, amoxicillin and levofloxacin were administered before breakfast and dinner, and bovine lactoferrin, which expresses its major effect at pH 6.0,⁴⁰ was given orally 2 h after breakfast (200 mg) and 2 h after dinner (100 mg), when esomeprazole was capable of reducing gastric acidity.⁴⁰ The two groups were treated with these therapeutic agents for 10 days.

H. pylori eradication was defined as a negative result for the UBT performed at least 8 weeks after the end of treatment with a delta-over-baseline value ≤5.

Statistical analysis

Statistical evaluation was carried out using χ^2 analysis. A *P* value of ≤0.05 was considered statistically significant.

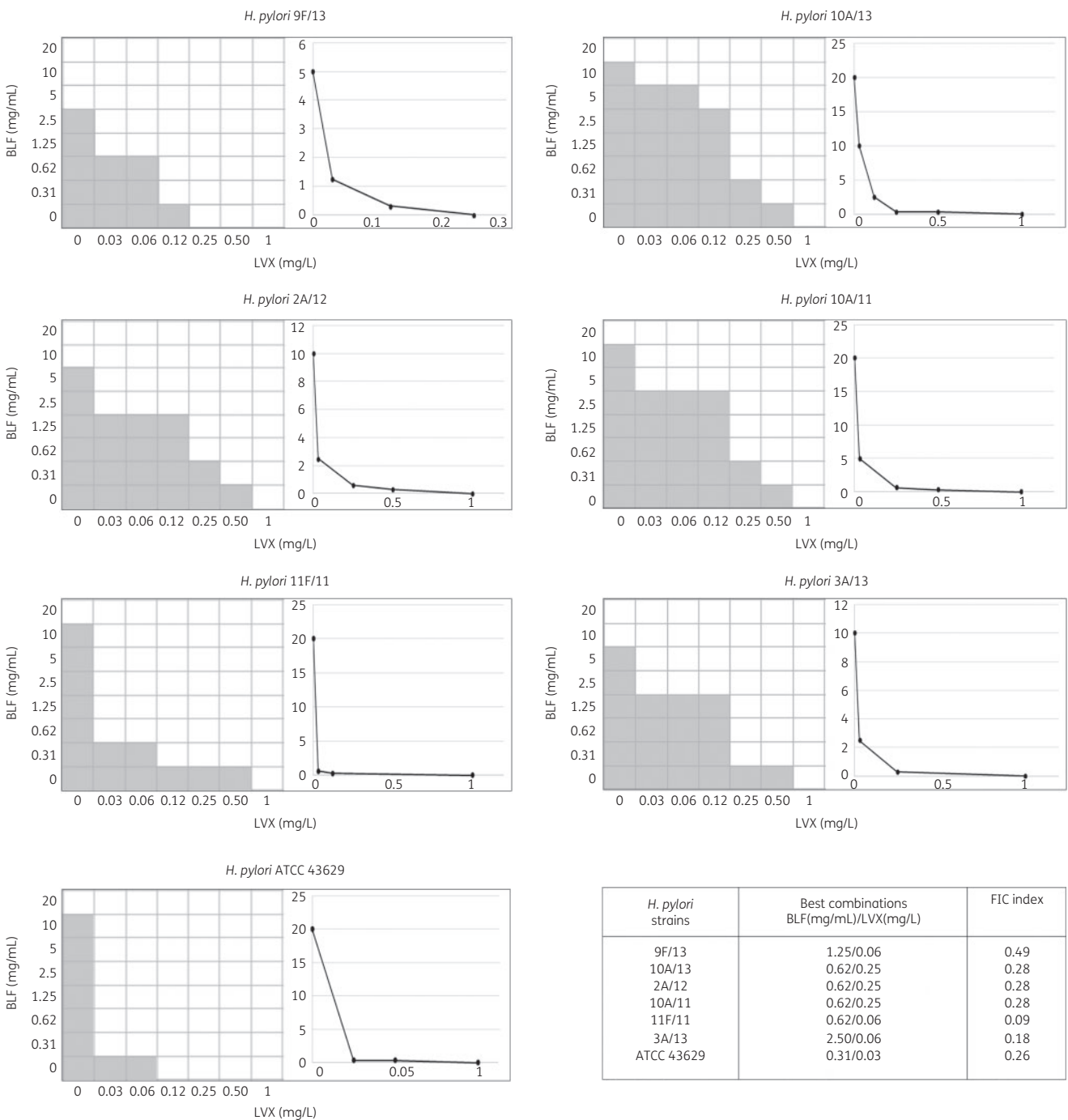


Figure 1. Chequerboard assays and isobolograms for evaluation of synergism between bovine lactoferrin (BLF) and levofloxacin (LVX) combinations against *H. pylori* strains. The calculation of the best BLF and LVX combination and the FIC index is displayed. FIC index values were interpreted as follows: synergy (FIC index ≤ 0.5); antagonism (>4.0); and no interaction ($>0.5-4.0$). Shading shows visible bacterial growth.

Results

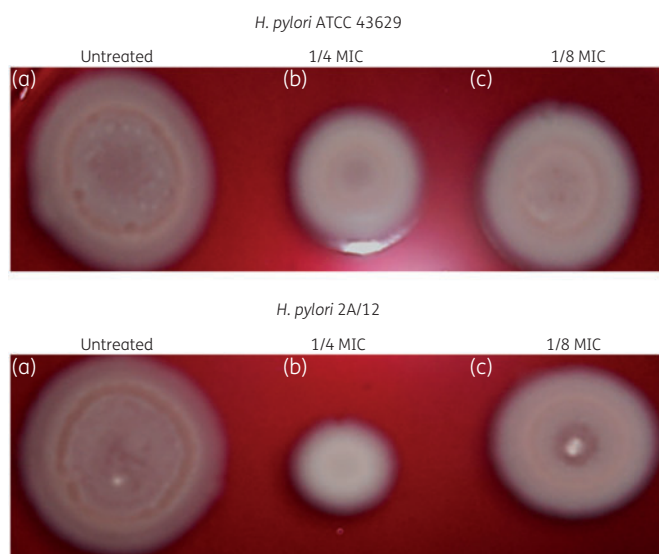
In vitro test of bovine lactoferrin against clinical *H. pylori* strains

The antimicrobial susceptibility panel and the main virulence markers of the *H. pylori* strains used in the experiments are shown in Table 1. Five out of six clinical strains displayed a resistance

profile with at least four drug resistances; among them, two clinical strains (*H. pylori* 10A/13 and *H. pylori* 2A/12) showed an MDR profile with resistance to three classes of antibiotics. All detected strains were *cagA*+, the *iceA1* allelic type was found in three strains and the main *vacA* genotype allelic combination was s1m1i1. No correlation was detected between antibiotic resistance profiles and virulence markers.

Table 2. Antibacterial activity of bovine lactoferrin against *H. pylori* strains

| Strain | MIC (mg/mL) | MBC (mg/mL) |
|------------|-------------|-------------|
| 9F/13 | 5 | 40 |
| 10A/13 | 20 | 80 |
| 2A/12 | 10 | 60 |
| 10A/11 | 20 | 40 |
| 11F/11 | 20 | 80 |
| 3A/13 | 10 | 40 |
| ATCC 43629 | 20 | 160 |

**Figure 2.** Representative images of sliding motility assay of *H. pylori* strains. (a) Control (ATCC 43629, diameter 19 ± 0.3 mm; 2A/12, diameter 21 ± 0.2 mm). (b) Strains cultured for 3 days and exposed to one-fourth MIC of bovine lactoferrin (BLF) (ATCC 43629, diameter 11 ± 0.1 mm; 2A/12, diameter 8 ± 0.1 mm). (c) Strains cultured for 3 days and exposed to one-eighth MIC of BLF (ATCC 43629, diameter 16 ± 0.3 mm; 2A/12, diameter 11 ± 0.1 mm).

The MICs and MBCs of bovine lactoferrin for *H. pylori* strains are shown in Table 2. Specifically, bovine lactoferrin inhibited all strains tested, with MIC values ranging from 5 to 20 mg/mL and MBC values ranging from 40 to 160 mg/mL. When bovine lactoferrin was tested in association with levofloxacin against the *H. pylori* strains the MIC values of levofloxacin fell significantly to a range of 0.03–0.25 mg/L, whereas for bovine lactoferrin the range was 0.31–2.5 mg/mL. Synergy was detected in all tested strains (FIC index ≤ 0.5) (Figure 1).

The checkerboard model and isobolograms for the combination of bovine lactoferrin and levofloxacin to define the synergy and to calculate the FIC index are shown in Figure 1. Synergy (FIC index ≤ 0.5) was detected in all strains tested, with a reduction of 4- to 16-fold in the MIC for levofloxacin. The best combinations of bovine lactoferrin and levofloxacin for each strain are indicated in Figure 1. For the *H. pylori* 11F/11 resistant strain, the FIC value was 0.09, with a reduction in the MICs of bovine lactoferrin and

Table 3. Summary of study data

| | Group A | Group B |
|--|--|--|
| Therapeutic trial: | 40 mg ESO, 1000 mg AMX, 500 mg LVX (all twice daily) | 40 mg ESO, 1000 mg AMX, 500 mg LVX, 100 mg BLF (all twice daily) |
| Number of patients | 24 | 53 |
| Gender, <i>n</i> (%) | | |
| female | 16 (66.6) | 32 (60.3) |
| male | 8 (33.4) | 21 (39.7) ^a |
| Age, years, mean (range) | 48 (23–70) | 50 (20–75) ^a |
| Smoking habit, <i>n</i> /N (%) | | |
| female | 9/16 (56.2) | 21/32 (65.6) |
| male | 3/8 (37.5) | 10/21 (47.6) ^a |
| Alcohol use ≤ 30 g/week, <i>n</i> /N (%) | | |
| female | 8/16 (50) | 20/32 (62.5) |
| male | 6/8 (75) | 16/21 (76.1) ^a |
| Dyspepsia, <i>n</i> (%) | 24 (100) | 53 (100) |
| Endoscopy, <i>n</i> /N (%) | 7/24 (29.1) | 16/53 (30.1) ^a |
| Gastritis, <i>n</i> /N (%) | 4/7 (57.1) | 10/16 (62.5) ^a |
| Duodenitis, <i>n</i> /N (%) | 2/7 (28.5) | 5/16 (31.2) ^a |
| Oesophagitis (Los Angeles, grade A), <i>n</i> /N (%) | 1/7 (14.2) | 3/16 (18.7) ^a |

ESO, esomeprazole; AMX, amoxicillin; LVX, levofloxacin; BLF, bovine lactoferrin.

^aNot significant versus Group A (χ^2 analysis).

levofloxacin of 16- and 32-fold, respectively. Antagonism was not observed.

The loss of *H. pylori* ATCC 43629 and 2A/12 motility in the presence of bovine lactoferrin at sub-MIC values is shown in Figure 2. When treated with bovine lactoferrin at one-fourth and one-eighth of MICs, all strains studied displayed a smaller diameter of growth on soft agar in comparison with the untreated strains. Specifically, the reduction was significant at one-fourth of the MIC of bovine lactoferrin ($P < 0.05$).

Therapeutic trial

Patient characteristics are summarized in Table 3. In Group A, after recruitment of 24 patients who completed the treatment and returned for follow-up, six failures (UBT positive) were recorded. At this point recruitment was halted as it was judged to be impossible to exceed the 80% success rate even if recruitment had continued up to 30 cases. The cure rate in this group was 75% in ITT and PP analyses (95% CI 57.68%–92.32%).

In Group B, 30 patients were initially recruited and 28 completed the study, whereas 2 patients were lost to follow-up. Two failures (UBT positive) and 26 successful eradications (UBT negative) were recorded with a success rate in PP analysis of 92.8%. Since a 93% success rate was not achieved as specified in the protocol, recruitment continued up to 53 patients (Figure 3). The final analysis in this group showed that 49 of the 53 patients recruited showed successful eradication, with a cure rate of 92.45% (95% CI 86.52%–100.38%) by ITT and 49/51 of (96.07%, 95% CI 90.62%–101.38%) by PP analysis.

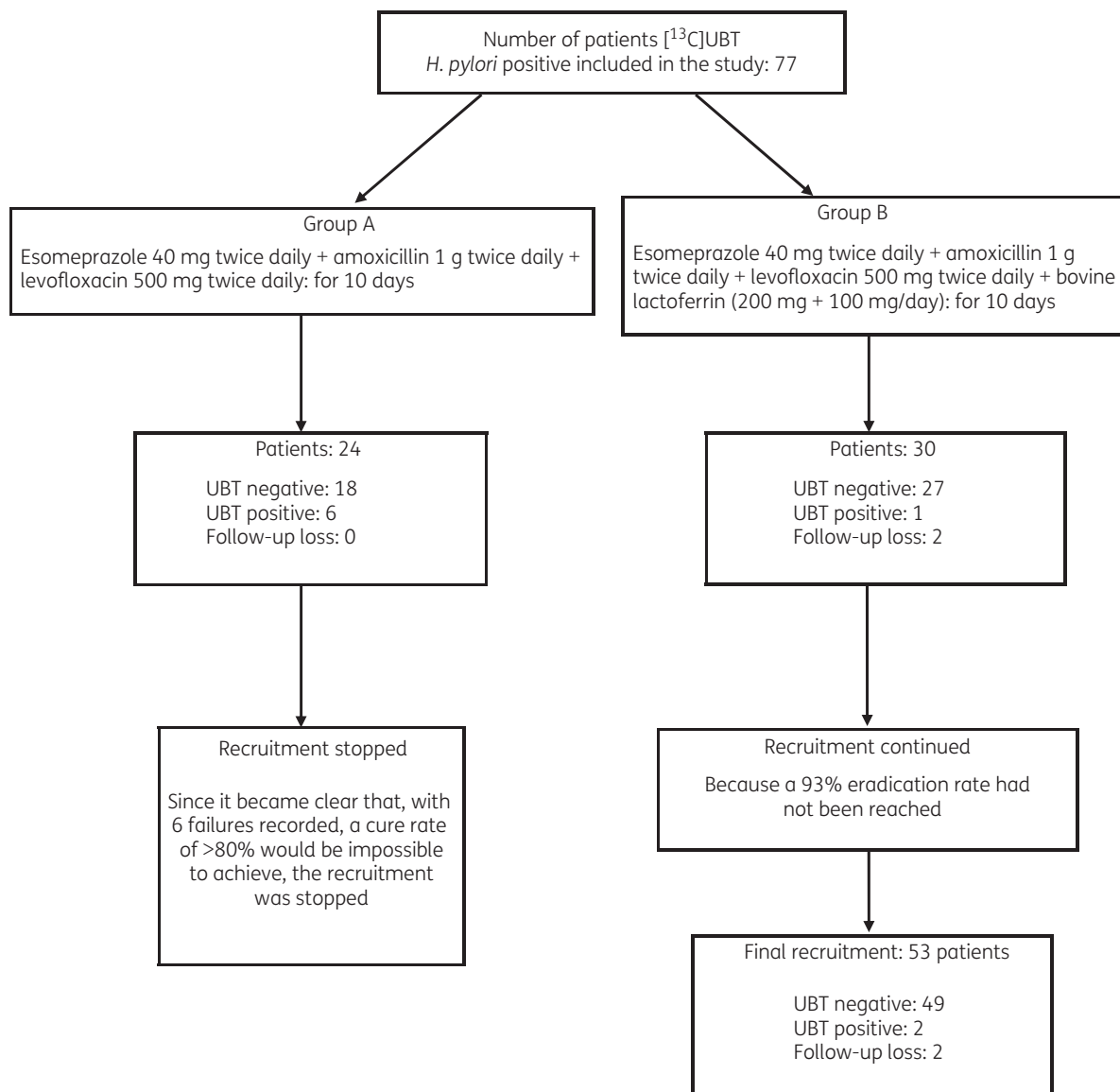


Figure 3. Flow diagram for eradication of *H. pylori*.

The χ^2 analysis of the Group A and Group B eradication rates showed a significant difference ($P = 0.0058$).

Adverse effects

Adverse events occurred in four patients from Group A (16.6%) and eight patients from Group B (15.09%) and were represented by diarrhoea [2 (8.3%) and 3 (5.6%), respectively], abdominal pain [1 (4.1%) and 3 (5.6%)] and nausea [1 (4.1%) and 2 (3.7%)]. These adverse effects did not induce a discontinuation of the treatment.

Discussion

This study demonstrates that bovine lactoferrin *in vitro* inhibits the growth and motility of *H. pylori*. When used in combination with levofloxacin against *H. pylori* strains resistant to levofloxacin and

other antibiotics, bovine lactoferrin at sub-MIC values restores the effectiveness of the antibiotic through a synergistic action.

In vivo, bovine lactoferrin achieves a therapeutic gain of 21% when added to a triple therapy with a proton pump inhibitor, levofloxacin and amoxicillin in a group of patients living in a geographical area where *H. pylori* resistance to levofloxacin is >40% of tested strains.

Bovine lactoferrin can improve the potency of traditional antimicrobial regimens by fighting antimicrobial resistance through the reduction of flagellar motility and consequently microbial colonization. Our data are in line with a recent study that emphasized an innovative strategy of using natural bioactive substances capable of synergizing with antibiotics and also expressing antivirulence activity to combat antibiotic resistance and bacterial virulence.⁴¹

The use of natural compounds combined with antibiotics represents an important strategy to tackle the antibiotic resistance

phenomenon.⁴² The synergistic action obtained reduces the MIC of levofloxacin, restoring the efficacy of the antimicrobial drug.

Bovine lactoferrin could also exert an antimicrobial effect against *H. pylori*⁴³ *in vitro* and *in vivo* by inhibition of its growth at pH 6.⁴⁰ In addition, the antibacterial activity of bovine lactoferrin may be attributed to its ability to bind iron with great affinity and prevent its utilization by the bacteria.

The evidence that deferoxamine, another iron chelator, inhibits *H. pylori* growth may support this hypothesis.^{44,45} Additional properties of bovine lactoferrin that may explain its antibacterial activity include immune-modulatory activity,⁴⁶ antioxidant activity⁴⁷ and a significant inhibitory effect on the *in vivo* attachment of *H. pylori* to the stomach, associated with a reduction in bacterial number and inflammation.⁴⁸ It has also been shown that recombinant human lactoferrin can bind and disrupt some bacterial cell membranes,^{49,50} and when it is co-administered with amoxicillin against Gram-negative bacteria it increases its efficacy, as found in animal models. Furthermore, in a recent study Yuan *et al.*⁵¹ examined the effectiveness of recombinant human lactoferrin isolated from transgenic goats as a treatment for *H. pylori in vitro* and *in vivo*. For the *in vitro* experiments, the results revealed that recombinant human lactoferrin not only inhibited the growth of *H. pylori*, but also suppressed the expression of two major virulence factors, *cagA* and *vacA*.

It has been shown that levofloxacin in combination with amoxicillin and proton pump inhibitors can be an effective regimen for first-line anti-*H. pylori* treatment.^{52,53} In a meta-analysis, it has been observed that levofloxacin-based first-line therapy and standard therapy have equivalent efficacy and safety profiles for eradication of *H. pylori*.⁵⁴ However, *H. pylori* resistance to levofloxacin has clearly increased in recent years;^{17,55} this may explain why the triple therapy that includes this antibiotic has reduced therapeutic efficacy. Bovine lactoferrin has already been shown to possibly potentiate the efficacy of a triple therapy that included antibiotics other than levofloxacin for the treatment of *H. pylori* infection. In a study by de Bortoli *et al.*,⁵⁶ the addition of bovine lactoferrin to a triple therapy with esomeprazole, amoxicillin and clarithromycin increased the success rate by 16% (ITT and PP analysis). In a similar study adding bovine lactoferrin to a therapy with rabeprazole, tinidazole and clarithromycin, Di Mario *et al.*⁵⁷ were able to increase the eradication rate by 21%, with total eradication in 92.2% (ITT analysis) and 95.2% (PP analysis) of treated cases. In our study the addition of bovine lactoferrin to a triple therapy that included esomeprazole, amoxicillin and levofloxacin for *H. pylori* infection significantly increased the eradication rate compared with that of the non-supplemented regimen, with a therapeutic gain of 21%.

Since resistance of *H. pylori* to levofloxacin in our region is present in >40% of tested strains it may be hypothesized that bovine lactoferrin potentiates the effect of levofloxacin, reducing the level of resistance of the bacterium and thereby increasing the overall efficacy, as shown in the *in vitro* study.

In addition, adding bovine lactoferrin to a levofloxacin-based triple therapy may avoid a levofloxacin preliminary susceptibility test, as it is when clarithromycin-based treatment is used in geographical areas with high clarithromycin resistance.¹⁰

The limitations of our study include the absence of a preliminary culture and susceptibility testing since *H. pylori* resistance to levofloxacin is quite high in our region. Moreover, as reviewed by Gisbert,⁵⁸ even after culture-guided rescue treatments the lowest

eradication rates were obtained in patients with *H. pylori* strains susceptible to all antibiotics, indicating that factors other than *in vitro* antibiotic susceptibility, such as biofilm *H. pylori* production, may influence eradication rates. However, we believe that the randomized allocation of patients to the two groups would have overcome this weakness to some extent.

Therefore, it may be concluded that the 10 day quadruple therapy consisting of esomeprazole, amoxicillin, levofloxacin and bovine lactoferrin can be generally used as the first-line treatment of *H. pylori* infection, including those regions where *H. pylori* expresses high resistance to fluoroquinolones.

Acknowledgements

We thank Laurino Grossi for his useful contribution to this study and Mrs Catherine Hlywka for reviewing the English style of the manuscript.

Funding

This study was supported by internal funding and Fondo di Ataneo per la Ricerca (FAR 2017) held by Leonardo Marzio and Luigina Cellini.

Transparency declarations

None to declare.

References

- 1 Ford AC, Gurusamy KS, Delaney B *et al.* Eradication therapy for peptic ulcer disease in *Helicobacter pylori*-positive people. *Cochrane Database Syst Rev* 2016; issue 4: CD003840.
- 2 Kuo SH, Cheng AL. *Helicobacter pylori* and mucosa-associated lymphoid tissue: what's new. *Hematol Soc Hematol Educ Program* 2013; **2013**: 109–17.
- 3 Bornschein J, Malfertheiner P. *Helicobacter pylori* and gastric cancer. *Dig Dis* 2014; **32**: 249–64.
- 4 Diaconu S, Predescu A, Moldoveanu A *et al.* *Helicobacter pylori* infection: old and new. *J Med Life* 2017; **10**: 112–7.
- 5 De Francesco V, Bellesia A, Ridola L *et al.* First-line therapies for *Helicobacter pylori* eradication: a critical reappraisal of updated guidelines. *Ann Gastroenterol* 2017; **30**: 373–9.
- 6 Vakil N. *H. pylori* treatment: new wine in old bottles? *Am J Gastroenterol* 2009; **104**: 26–30.
- 7 Kaur I. Novel strategies to combat antimicrobial resistance. *J Infect Dis Ther* 2016; **4**: 2–6.
- 8 Kim JS, Park SM, Kim BW. Sequential or concomitant therapy for eradication of *Helicobacter pylori* infection: a systematic review and meta-analysis. *J Gastroenterol Hepatol* 2015; **30**: 1338–45.
- 9 Calvet X, Lopez-Lorente M, Cubells M *et al.* Two-week dual vs. one-week triple therapy for cure for *Helicobacter pylori* infection in primary care: a multi-centre, randomized trial. *Aliment Pharmacol Ther* 1999; **13**: 781–6.
- 10 Malfertheiner P, Megraud F, O'Morain CA *et al.*; European *Helicobacter* and Microbiota Study Group and Consensus panel. Management of *Helicobacter pylori* infection—the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6–30.
- 11 Perez Aldana L, Kato M, Nakagawa S *et al.* The relationship between consumption of antimicrobial agents and the prevalence of primary *Helicobacter pylori* resistance. *Helicobacter* 2002; **7**: 306–9.

- 12 Megraud F, Coenen S, Versporten A *et al.*; Study Group participants. *Helicobacter pylori* resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut* 2013; **62**: 34–42.
- 13 De Francesco V, Giorgio F, Hassan C *et al.* Worldwide *H. pylori* antibiotic resistance: a systematic review. *J Gastrointest Liver Dis* 2010; **19**: 409–14.
- 14 Megraud F. *H. pylori* antibiotic resistance: prevalence, importance and advances in testing. *Gut* 2004; **53**: 1374–84.
- 15 Boyanova L, Mitov I. Geographic map and evolution of primary *Helicobacter pylori* resistance to antibacterial agents. *Expert Rev Anti Infect Ther* 2010; **8**: 59–70.
- 16 Di Giulio M, Di Campli E, Di Bartolomeo S *et al.* *In vitro* antimicrobial susceptibility of *Helicobacter pylori* to nine antibiotics currently used in Central Italy. *Scand J Gastroenterol* 2016; **3**: 263–9.
- 17 Huang JQ, Hunt RH. Treatment after failure. The problem of 'non-responders'. *Gut* 1999; **45**: 140–4.
- 18 Cuadrado-Lavín A, Salcines-Caviedes JR, Carrascosa MF *et al.* Levofloxacin versus clarithromycin in a 10 day triple therapy regimen for first-line *Helicobacter pylori* eradication: a single-blind randomized clinical trial. *J Antimicrob Chemother* 2012; **67**: 2254–9.
- 19 Hung IF, Chan P, Leung S *et al.* Clarithromycin amoxicillin-containing triple therapy: a valid empirical first-line treatment for *Helicobacter pylori* eradication in Hong Kong? *Helicobacter* 2009; **14**: 505–11.
- 20 Molina-Infante J, Perez-Gallardo B, Fernandez-Bermejo M *et al.* Clinical trial: clarithromycin vs. LEV in first-line triple and sequential regimens for *Helicobacter pylori* eradication. *Aliment Pharmacol Ther* 2010; **31**: 1077–84.
- 21 Iacopini F, Crispino P, Paoluzi OA *et al.* One-week once-daily triple therapy with esomeprazole, LEV and azithromycin compared to a standard therapy for *Helicobacter pylori* eradication. *Dig Liver Dis* 2005; **37**: 571–6.
- 22 Malfertheiner P, Bazzoli F, Delchier JC *et al.*; Pylera Study Group. *Helicobacter pylori* eradication with a capsule containing bismuth citrate potassium, metronidazole, and tetracycline given with omeprazole versus clarithromycin-based triple therapy: a randomized, open-label, non inferiority phase 3 trial. *Lancet* 2011; **377**: 905–13.
- 23 Ciccaglione AF, Cellini L, Grossi L *et al.* A triple and quadruple therapy with doxycycline and bismuth for first-line treatment of *Helicobacter pylori* infection: a pilot study. *Helicobacter* 2015; **20**: 390–6.
- 24 Sachdeva A, Rawat S, Nagpal J. Efficacy of fermented milk and whey proteins in *Helicobacter pylori* eradication: a review. *World J Gastroenterol* 2014; **20**: 724–37.
- 25 Jenssen H, Hancock RE. Antimicrobial properties of lactoferrin. *Biochimie* 2009; **91**: 19–29.
- 26 Ellison RT, Giehl TJ, LaForce FM. Damage of the outer membrane of enteric Gram-negative bacteria by lactoferrin and transferrin. *Infect Immun* 1988; **56**: 2774–81.
- 27 Yamazaki N, Yamuchi K, Kawase K *et al.* Antibacterial effects of lactoferrin and a pepsin-generated lactoferrin peptide against *Helicobacter pylori* *in vitro*. *J Infect Chemother* 1997; **3**: 85–9.
- 28 Wada T, Aiba Y, Shimizu K *et al.* The therapeutic effect of bovine lactoferrin in the host infected with *Helicobacter pylori*. *Scand J Gastroenterol* 1999; **34**: 238–43.
- 29 Okuda M, Nakazawa T, Yamauchi K *et al.* Bovine lactoferrin is effective to suppress *Helicobacter pylori* colonization in the human stomach: a randomized, double-blind, placebo-controlled study. *J Infect Chemother* 2005; **11**: 265–9.
- 30 Guttner Y, Windsor HM, Viiala CH *et al.* Human recombinant lactoferrin is ineffective in the treatment of human *Helicobacter pylori* infection. *Aliment Pharmacol Ther* 2003; **17**: 125–9.
- 31 Cellini L, Grande R, Di Campli E *et al.* Analysis of genetic variability, antimicrobial susceptibility and virulence markers in *Helicobacter pylori* identified in Central Italy. *Scand J Gastroenterol* 2006; **41**: 280–7.
- 32 Grande R, Di Giulio M, Di Campli E *et al.* A model of *Helicobacter pylori* persistence in a case of gastric cancer. *New Microbiol* 2010; **33**: 343–9.
- 33 Clinical and Laboratory Standards Institute. *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria—Third Edition: M45*. CLSI, Wayne, PA, USA, 2016.
- 34 Magi G, Marini E, Facinelli B. Antimicrobial activity of essential oils and carvacrol, and synergy of carvacrol and erythromycin, against clinical, erythromycin-resistant Group A streptococci. *Front Microbiol* 2015; **6**: 1–7.
- 35 Cellini L, Di Campli E, Masulli M *et al.* Inhibition of *Helicobacter pylori* by garlic extract (*Allium sativum*). *FEMS Immunol Med Microbiol* 1996; **13**: 273–7.
- 36 Odds FC. Synergy, antagonism, and what the checkerboard puts between them. *J Antimicrob Chemother* 2003; **52**: 1.
- 37 Roncarati D, Danielli A, Spohn G *et al.* Transcriptional regulation of stress response and motility functions in *Helicobacter pylori* is mediated by *HspR* and *HrcA*. *J Bacteriol* 2007; **189**: 7234–43.
- 38 Tsang J, Hoover TR. Requirement of the flagellar protein export apparatus component FliO for optimal expression of Flagellar Genes in *Helicobacter pylori*. *J Bacteriol* 2014; **15**: 2709–17.
- 39 Graham DY. Efficient identification and evaluation of effective *Helicobacter pylori* therapies. *Clin Gastroenterol Hepatol* 2009; **7**: 145–8.
- 40 Dial EJ, Hall LR, Serna H *et al.* Antibiotic properties of bovine lactoferrin on *Helicobacter pylori*. *Dig Dis Sci* 1998; **43**: 2750–6.
- 41 Marini E, Di Giulio M, Magi G *et al.* Curcumin, an antibiotic resistance breaker against a multiresistant clinical isolate of *Mycobacterium abscessus*. *Phytother Res* 2018; **32**: 488–95.
- 42 Brown D. Antibiotic resistance breakers: can repurposed drugs fill the antibiotic discovery void? *Nat Rev Drug Discov* 2015; **14**: 821–32.
- 43 Sachdeva A, Nagpal J. Meta-analysis: efficacy of bLF in *Helicobacter pylori* eradication. *Aliment Pharmacol Ther* 2009; **29**: 720–30.
- 44 Sanchez L, Calvo M, Brock J. Biological role of lactoferrin. *Arch Dis Child* 1992; **67**: 657–61.
- 45 Illingworth D, Walter K, Griffiths P *et al.* Siderophore production and iron-regulated envelope proteins of *Helicobacter pylori*. *Zentralbl Bakteriol* 1993; **280**: 113–9.
- 46 Singh PK, Parsek MR, Greenberg EP *et al.* A component of innate immunity prevents bacterial biofilm development. *Nature* 2002; **417**: 552–5.
- 47 Baldwin DA, Jenny ER, Aisen P. The effect of human serum transferrin and milk lactoferrin on hydroxyl radical formation from superoxide and hydrogen peroxide. *J Biol Chem* 1984; **259**: 13391–4.
- 48 Borody TJ, Ashman O. Lactoferrin: milking ulcers? *Dig Liver Dis* 2003; **35**: 691–3.
- 49 Mielke S, Reddy R, Osato MS *et al.* Direct activity of recombinant, human lactoferrin against *Helicobacter pylori*. *J Clin Microbiol* 1996; **34**: 2593–4.
- 50 Dial EJ, Romero JJ, Headon DR *et al.* Recombinant human lactoferrin is effective in the treatment of *Helicobacter felis*-infected mice. *J Pharm Pharmacol* 2000; **52**: 1541–6.
- 51 Yuan Y, Wu Q, Cheng G *et al.* Recombinant human lactoferrin enhances the efficacy of triple therapy in mice infected with *Helicobacter pylori*. *Int J Mol Med* 2015; **36**: 363–8.
- 52 Nista EC, Candelli M, Zocco MA *et al.* LEV-based triple therapy in first-line treatment for *Helicobacter pylori* eradication. *Am J Gastroenterol* 2006; **101**: 1985–90.
- 53 Telaku S, Manxhuka-Kerliu S, Kraja B *et al.* The efficacy of LEV-based triple therapy first-line *Helicobacter pylori* eradication. *Med Arch* 2013; **67**: 348–50.
- 54 Peedikayil MC, AlSohaibani FI, Alkhenizan AH. LEV-based first-line therapy versus standard first-line therapy for *Helicobacter pylori* eradication: meta-analysis of randomized controlled trials. *PLoS One* 2014; **9**: e85620.
- 55 Marzio L, Cellini L, Amitrano M *et al.* *Helicobacter pylori* isolates from proximal and distal stomach of patients never treated and already treated show

genetic variability and discordant antibiotic resistance. *Eur J Gastroenterol Hepatol* 2011; **23**: 467–72.

56 de Bortoli N, Leonardi G, Ciancia E et al. *Helicobacter pylori* eradication: a randomized prospective study of triple therapy versus triple therapy plus lactoferrin and probiotics. *Am J Gastroenterol* 2007; **102**: 951–6.

57 Di Mario F, Aragona G, Dal Bó N et al. Use of lactoferrin for *Helicobacter pylori* eradication. *Dig Liv Dis* 2003; **35**: 706–10.

58 Gisbert JP. “Rescue” regimens after *Helicobacter pylori* treatment failure. *World J Gastroenterol* 2008; **14**: 5385–402.