Fermented Soybean Liquid Alleviated Peptic Ulcer Through Destroying Acidic Proton–Pump Rather Than Suppressing Urease of *Helicobacter pylori* — A Kinetic Analysis

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ABSTRACT

Fermented soybean liquid (FSL) has been well cited for its broad spectrum of biological effects, yet its documented gastropeptic ulcer (GPU) ameliorating effect is still lacking. We hypothesize that to avoid the injury exerted by gastric fluid, HP has to be sheltered in chyme emulsions immediately on infection. The HP urease (HPU) and the acidic proton pump (PP) may act as the “Two-Point pH Modulator” to maintain an optimum pH between 6-7, and FSL is able to destroy such modulating mechanism. FSL exhibited higher contents of isoflavonoids (2.5-17.3 folds) and essential amino acids (1.5-4.0 folds) than the non-fermented. FSL administered 1g/20 mL t.i.d. for three months eradicated *Helicobacter pylori* (HP) by 82% in 37 volunteers having GPU (p<0.20), simultaneously the plasma conjugated diene and TBARs levels were significantly resumed (p< 0.05). Kinetic analysis based on the conventional ”Urease Theory” revealed that a cluster of 2.0×10^9 of HP cells is required for a single attack in the gastric lumen at pH 1.0-2.5. To verify our hypothesis, the Chyme-Shelter Testing was conducted in artificial gastric fluid (pH 2.4±0.20). Results showed the HP cell viability was time- and size-dependent. At 20 min of contact time, the viability was 100, 4.2, 31.4, 43.3, 57.2, and 82.6% respectively in the intact-, dispersed-, and the particulate chymes (mesh size #80, #60, #40 and #20). The corresponding data became 96.2, 0.0, 14.5, 18.5, 21.3, and 28.6%,

respectively at a contact time of 40 min. Conclusively, the kinetic analysis and the Chyme-Shelter Testing revealed that direct infection by bare HP cells is unlikely possible in real status. FSL is beneficial to GPU most probably due to its blood alkalinity-raising capability to destroy the PP and its ROS suppressing effect.

Keywords: fermented soybean liquid; *H. pylori* urease; peptic ulcer; isoflavonoids; proton pump

INTRODUCTION

Accumulating evidences have linked the presence of *Helicobacter pylori* with development of gastritis and peptic ulcer (PU) (*1*). *H. pylori* resides primarily in the gastric mucosa without invading the gastric epithelium, causing persistent low-grade gastric inflammation (*2*). Normally, colonization of *H. pylori* in the duodenum is restricted to areas of gastric metaplasia and metaplastic gastric epithelium in the duodenal bulb of most patients with PU (*2*). Currently we have recognized more than 90% of duodenal ulcers and 80% of gastric ulcers result from infection (*2*). The prevalence of PU can be caused by i) weakened intrinsic mucosal defensive factors; ii) low protein diet supplement; iii) increased gastric secretion. These aggressive factors can enhance the invasive capability of *H. pylori*, promoting free radical attack and enhancing secretion of gastric acid, pepsin, bile salt, polypeptides, and hypercalcemia, leading to antral distension and alkalination (Private communication from the gastroenterologist, Prof. Pan, Taipei Medical University, Taiwan). When the radical attacks synergized by some atherosclerotic risk factors like smoking, alcoholic drinking, high fat diet, lacking exercise, or high brain stress, could emerge to elicit in vivo peroxidation of low density lipoprotein (LDL), leading to accumulation of oxidized LDL (ox-LDL) in plasma (*3*). Ox-LDL in turn can trigger a serial formation of inflammatory cytokines like tumor necrosis factor (TNF), interleukin-1, -4, and interferon-γ, resulting in aggravation of gastric ulcer (*4*).

95 Current treatments for *H. pylori* are becoming less effective due to mounting antibiotic
96 resistance. Novel strategy suggests a treatment course of 7 to 14 days by "Triple
97 therapy", which usually comprises a proton-pump inhibitor and 2 antimicrobial agents,
98 apparently more effective than the conventional "Dual therapy" (*5*).

99 Soybean, *Glycine max* Merill, belongs to family *Leguminosae*. It has been cultivated
100 and utilized as foods, nutrition, and medicine in daily lives of Chinese for a history
101 longer than 5,000 years. Soy bean proteins (SBP) are a bile acid secretagogue, a
102 hypolipidemic, as well as a strong LDL receptor activator (*6*). Dietary protein peptic
103 hydrolysate stimulated cholecystokinin release. Cholecystokinnin secretion induced
104 gastroprotection through the aid of nitric oxide and blood flow increase (*6*). Moreover,
105 FSL contains significantly increased amount of unique phytochemicals, isoflavonoids
106 especially genistein, daidzein (*7*), which are more beneficial to health.

107 The therapeutic effect of FSL on PU is still lacking. We hypothesize that FSL may
108 enhance intrinsic mucosal defensive factors by providing sufficient protein, bioactive
109 peptides and amino acid supplement through increased blood flow, alleviating ROS
110 induced by insulin resistance and PU, suppressing hypercholesterolemic and
111 hypertriglyceridemic risk factors, activating some antioxidative signaling pathway and
112 modifying the micro acidic environment that otherwise would be favorable to
113 proliferation and invasiveness of *H. pylori* (Fig. 1). To confirm this, we compared the

constituents before and after fermentation, and conducted this experiment with FSL on 37 pioneers exhibiting symptoms of PU.

MATERIALS AND METHODS

Chemicals

Genistein and genistin were isolated from a concentrate prepared from soy molasses by Protein Technologies International (St. Louis, MO, USA) as directed by the manufacturer. Soybean lecithine and daidzein was purchased from LC Labs (Woburn, MA, USA). Biochannin A, equol, dihydrodaidzein, O-demethylangolensin (ODMA), enterolactone, enterdiol and other chemical used were provided by Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

Strains

Aspergillus oryzae (Koji), *Saccharomyces rouzii*, and *Pedicoccus halophilus* were purchased from the Bioresource Collection and Research Center (BCRC) of the Food Industrial Research and Development Institute (Hsin-Chu City, Taiwan). The isolate of *H. pylori* (strain ATCC 43579, originated from human gastric samples) was obtained from the American Type Culture Collection (Manassas, VA).

Plate Cultivation of *H. pylori*

H. pylori was cultivated by method described by Kehler et al. (*8*). Briefly, Brucella broth with CO_2 plus SPS (sodium polyanetholsulfonate) (Becton Dickinson Microbiology Systems, Cockeysville, Md. USA) was used for cultivation of *H. pylori*. Control organisms (*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923) were used for all blood culture systems. All isolates that had been stored at -70 ^{o}C were thawed 3 to 4 days before inoculation. *H. pylori* was first plated on fresh *H. pylori* agars (89% Brucella agar, 10% defibrinated calf blood plus 1% IsoVitaleX) and incubated in a microaerobic atmosphere (CampyPak, BBL Microbiology system). Control strains were plated onto sheep blood and chocolate agar plates 1-2 days before inoculation (Remel, Lenexa, Kans. USA). Bacterial inocula were prepared by suspending a few colonies in phosphate–buffered saline (PBS) to a density of a 1.0 McFarland Standard. Ten fold dilutions were made, and 500 µL of a 10^{-5} dilution was inoculated into each flask. The size of each inoculation was determined by colony counts in triplicate, and the colony counts were averaged. Each blood culture bottle was also inoculated with 5 mL of fresh human blood from one of the healthy volunteers who had been determined to be *H. pylori* antibody negative. The blood culture bottles were ventilated according to the manufacturer's instruction to mimic clinical conditions and incubated under aerobic conditions at 37^{o}C, high relative humidity (RH ≥ 85%), and 5% CO_2. After inoculation, each bottle was routinely

subcultured on day 1, 2, 3, 7, and day 14. 100 μL of broth was plated onto commercially available cholcolate and 5% sheep blood agars (Remel). Growth was recorded as positive if at least one colony of *H. pylori* was detected on any of the agars. *H. pylori* strains were further identified by morphology, and the identities were confirmed by positive urease, catalase, and oxidase tests. Control strains were identified by colony morphology, and the identities were confirmed by Gram staining, showing either Gram-positive cocci in clusters or Gram-negative rods. The actual growth in each blood culture bottle was performed for 72 h incubation. The smears of culture were plated onto the *H. pylori* plates (Sigma Aldrich, MO, UA), and incubated in CampyPak jars. The results were expressed as CFU/mL of broth. Geometric means for each time point were calculated by using Statgraphics 3.0 software (Graphics Software Systems, Inc., Rockville, MD, USA).

Fermentation Brine

The fermentation brine consisted of (in g/L) NaCl 2.5, KCl 2.5, $Ni(NO_3)_2$ 0.01, $Co(NO_3)_2$ 0.01, $MgSO_4$ 0.2, $CaCl_2 \cdot 2H_2O$, 0.3, $FeCl_2$ 0.1, Na_2HPO_4 0.5, KH_2PO_4, $(NH_4)_2SO_4$ 1.5, $ZnSO_4$ 0.1, $CuSO_4 \cdot 5H_2O$ 0.01. The brine was ajusted to pH 6.2 and autoclaved for 20 min at 121^{o}C.

Preparation of Fermented Soybean Liquid

169 *Glycine max* Merill (soybean) (SB) (4 kg), purchased from the local market of
170 Taichung, were rinsed with distilled water twice, each time for 5 min to remove the
171 adhering dust, then the soybean was steamed to remove microorganisms that adhere to
172 the surface. The sterilized soybeans were smashed and ground into pasty mass and the
173 fermentation brine was added at a ratio 1.5 L/kg soybean and mixed well. The final
174 volume was adjusted to 10 L with the sterilized brine. Koji, or *Aspergillus oryzae*, was
175 inoculated at 5.0g/L soybean. The process temperature was controlled within 33±2 ^{o}C
176 with pH set at 6.0±0.5. The starter fermentation was carried out for 5 days, then
177 *Saccharomyces rouzii* 3.0g/L and the bacterium *P. halophilus* 1.5g/L were
178 simulataneously inoculated into the fermentation liquid. The fermentation was
179 continued for another 10 days at 30 ^{o}C and pH 5.1. The product was diluted with 20%
180 fructose syrup to 50g/L to make FSL strength equivalent to 1.0g/20mL (FSL).

181 **Analysis of Isoflavonoids by HPLC-MS**

182 The isoflavonoids in sample ground SB paste and FSL were extracted with 10
183 fold-volume of 80% aqueous methanol (10 mL/g) as instructed by Barnes et al. (*9*).
184 The extracts were repeated thrice and the combined extracts were filtered with a
185 Whatman No. 2 filter paper. The filtrate was lyophilized and redissolved in deionized
186 water while keeping warming on a 45 ^{o}C water bath. The solution was subjected to
187 enzymic hydrolysis using mixed glucuronidase/sulfatase (Sigma Aldrich, MO, USA).

188 After incubated overnight at 37 ^{o}C, the aglycones were recovered by extraction with
189 diethyl ether (IFVex). All analyses were analyzed by HPLC (Hewlett Packard,
190 Wilmington, DE, USA, model 1050) linked to the PE-Sciex (Concorde, Ontario,
191 Canada) API III triple quadrupe mass spectrometer. Briefly, an Aquapore C_8
192 reversed-phase HPLC column (10 cm × 4.6 mm i.d. 300Å pore size) was used for
193 separation of isoflavonoids and phytoestrogens in SB and FSL. The mobile elution
194 was operated with a linear gradient of 0%-50% acetonitrile in 10 mM ammonium
195 acetate at a flow rate of 1 mL/min at pH 6.5 over 10 min. For mass determination,
196 samples were introduced into the mass spectrometer via the HN-APCI interface
197 operating in both the positive and negative modes. IFVex samples were separated by
198 reversed-phase HPLC column (ℓ× i.d.=15cm × 0.21cm). Brownlee Aquapore C_8
199 column (Varian Walnut Creek, CA, USA) using a linear 0%-50% gradient (5%/min)
200 of acetonitrile in 10 mM ammonium acetate at pH 6.5 and a flow rate of 0.2 mL/min.
201 The column elute was performed with a split 1:1, one stream passed into the Ion
202 Spray interface of the mass spectrometer operating in the negative ion mode, with an
203 orifice potential of -60 V in the full scan mode, ions entering the mass spectrometer
204 were analyzed over a m/z range from 50-800. MS-MS daughter ion spectra were
205 obtained by passing the molecular ions selected by the first quadrupole into an argon
206 gas collision cell, and the fragment ions were analyzed in a third quadrupole. Multiple

reaction ion monitoring (MRM) was carried out in a similar manner to MS-MS by selection of specific ions not only in the first quadrupole, but also in the third. Integration of peak area was carried out using the program MacQuan, provided by the mass spectrometer manufacturer (Hewlett Packard). Areas were corrected by the peak area of the added internal standard biohannin A and compared to the areas of a set of known isoflavonoid standards in order to quantify the content of isolflavonoids in the IFVex samples (*9*).

Amino Acid Analysis

The analysis of amino acids was performed as previously reported (*10*). Briefly, 1 g of soybean was smashed and ground to paste, to which 5 mL phosphate buffer (pH 6.8) was added. The solution was heated at 80 ^{o}C for 10 min. The extract was filtered through a Whatman No. 2 filter paper. The extraction was repeated three times. The filtrates were combined and made to 20 mL (SBE). Samples SBE and FSL, each 100 μL, were transferred into a 2 mL reaction vessel, to which 2 mL of 6 M HCl was added. The dissolved oxygen was purged off by nitrogen blowing for 10 min. The vessel was sealed, placed in the derivatization reactor, and heated at 110 ^{o}C for 24 h until the peptide moiety was completely hydrolyzed. The hydrolyzed product was lyophilized. The desiccated product was re-dissolved in 0.3 mL of 0.01 M HCl to obtain the sample amino acid mixture (AM).

Derivatization and Extraction

The authentic (0.3 mL) and AM (0.6 mL) solutions were respectively placed into a 3 mL-reaction vessels, to which the internal standard solution of norleucine (0.01 mL, 10 mg/mL) was added. After vigorous agitation, 0.1 mL of ethyl chloroformate and 1 mL of alcohol-pyridine were added and mixed thoroughly. To the mixture 2 mL of chloroform were added. The solution was agitated for 1 min to accelerate derivatization and extraction, 0.7 mL of water was added and shaken well. The reaction mixture was left to stand for 5 min to facilitate phase separation. The supernatant was discarded. The lower layer (i.e. the chloroform layer) was transferred into a new tube and dehydrated with 0.1g of anhydrous sodium sulfate. The dehydrated chloroform extract was transferred into the sample vessel and analyzed by GC/MS (*10*).

GC/MS Analysis

The GC/MS chromatography (Angilent 6890, Wilmington, DE, USA) installed with an FID detector and a column HP-5MS (ℓ×i.d. = 30 m× 0.25 mm; film thickness, 0.25 μm) was used for GC/MS analysis. The operation conditions were: flow rate of carrier nitrogen gas at 0.8 mL/min; the operation temperature of detector FID, 305 ^{o}C; that of injection port, 300 ^{o}C). The elution temperature was programmed initially at 50 ^{o}C for 1 min, then raised at an elevation rate 10 ^{o}C/min to 300 ^{o}C and held at which

for 6.5 min (*10*).

Detection of *H. pylori* by Non-Invasive Stool Antigen Test

The experimental procedure was performed according to Koletzko et al. (2003) with slight modification. Patients scheduled for endoscope were asked to bring a stool sample of their own at the time of the procedure or to send it within three days after endoscope before any therapy was initiated. One hundred mg of feces (corresponding to 2–5 pellets, depending on the size) were collected and dissolved in 1 mL 0.2% Tween 80 (Sigma-Aldrich, Bornem, Belgium) solution. The samples were vortexed vigorously until all fecal pellets were homogeneously suspended. After 10 minutes of sedimentation, the supernatant was first transferred to a 1.5 mL tube and centrifuged at 3800×g for 1 min, and then the supernatant was transferred to a new 1.5 mL tube and centrifuged at 20800×g for 5 minutes. The diluted stool supernatants (DSS) were collected and stored at -20 °C until analyzed. The stool antigen test was performed according to the manufacturer's recommendations using two different production lots. Those performing and reading the test were unaware of the *H pylori* status of the patients tested. The stool antigen test is an enzyme immunoassay (EIA) which uses monoclonal mouse anti-*H pylori* antibodies (Sigma Aldrich, MO, USA) adsorbed to micro wells as capture antibody. Firstly, 50µL of supernatant of the DSS and thereafter 50µL conjugated monoclonal antibody solution were added to the wells and

incubated for one hour at room temperature on a shaker. Unbound material was removed by washing four times with a washing buffer. After washing, 100 μL of a substrate solution were added and incubated for 10 minutes. Then 100 μL of the termination solution was added and the optical density was read by spectrophotometer at 450/630 nm dual wavelength. According to the manufacturer's guidelines, an optical density (OD) <0.150 is defined as a negative and an OD ≥0.150 as a positive test result. The test yielded a sensitivity of 98% and a specificity of 99% (*11*).

Subjects

This experimentation was approved by the Human Medical Experiment and Ethics Committee of Human Experiment in Hungkuang University according to the Declaration of Helsinki (1979), and informed consent was obtained from each patient, if appropriate. In the beginning, fifty seven pioneers were collected. The exclusive conditions were i) alcoholics, ii) smokers, iii) people who are taking NSAIDs, iv) night workers; v) cardiac diseased; vi) chronic kidney diseased; vii) psychological disturbances; viii) blood pressure systolic>160 mmHg; diastolic>110 mmHg. The inclusive requirements were 1) a positive breath test of *H. pylori*. In all 37 patients, the stool test instructed by Koletzko et al. (*11*) was performed instead of the conventional test method for *H. pylori* status which is carried out by biopsy based methods (rapid urease test, culture, and histology) and/or UBT. Pioneers were

283 excluded: i) if they had taken antibiotic or acid suppressive drugs (proton pump
284 inhibitors, H2 receptor antagonists, antacids, bismuth preparations) within four weeks
285 prior to testing, if they had received previous anti-*H. pylori* therapy, or if the *H. pylori*
286 status was not clearly defined (*11*), ii) normal office hour workers, iii) moderately
287 stressed by their work, iv) daily lunch hour not definite due to too heavy work loading,
288 v) A stage before blood stasis, i.e. chronic ulcer (stage Ul-II-IV), vi) age 30-55. Thus
289 the final attendants were only 37 subjects. The placebo group comprising 16 members
290 was given vitamin B complex one tablet u.i.d. only. The FSL-treated group having 21
291 members was given FSL orally at 20 mL t.i.d.

292 **Sera**

293 The blood samples of the two groups were collected by venipuncture bleeding at
294 month 0, 1, 2, and 2.5. The sample blood obtained was immediately centrifuged at
295 3000 rpm for 10 min. the supernatant was separated and stored at –20 ^{o}C before use
296 (Plasma sample, PS). The LDL was separated as described in the following section.

297 **Separation of LDL**

298 The LDL samples were prepared according to modified Yamanaka et al. (*3*). The LDL
299 fraction exhibiting shining golden yellow color was obtained. For use in this study,
300 the LDL was further dialyzed as directed. The final LDL obtained was determined for
301 its protein content. Bovine serum albumin (BSA) was used as the reference standard

to establish the calibration curve. The BioRad protein agent was used for protein determination. On dilution to 8 folds, the diluted reagent (1 mL) was added to 2 μL of LDL, on standing for 5 min, the absorbance was taken at 595 nm. The content of LDL was calculated against the BSA calibration curve. The remaining LDL was nitrogen gas filled and wrapped with aluminum foil to avoid direct light irradiation and stored at –20 ^{o}C. This LDL sample lasts fresh for 1 week.

Detection of the Oxidative Status in LDL

Determination of Conjugated Dienes

The dialyzed LDL was adjusted with PBS for its content to make a concentration of LDL 125μg protein/mL. The absorbance was read at 232 nm to examine the conjugated diene (CD) formation. The reference molar absorptivity of CD is ε_{234} = 2.95×10^{4} M^{-1} cm^{-1} (*3*).

Determination of TBARs

The dialyzed LDL was adjusted with PBS for its LDL content to LDL 125 μg protein/mL. The following procedures were performed according to Hsieh et al. (*3*). Finally, the supernatant was separated and the optical density was read at 532 nm. MDA was used as the reference compound, which on reaction with TBA has a molar extinguishing coefficient at 532 nm ε_{532} = $1.56\times10^{5}M^{-1}$ cm^{-1} (*3*).

Chyme-Shelter Testing

Preparation of Artificial Saliva (ASL)

To 40 mL of 0.05 M Mes-Tris buffer (pH 8.2), 50 mg of mucin and 50 μL of thermostable R-amylase (EC 3.2.1.1, A3306, Sigma-Aldrich, St. Louis, MO) was added (ASL).

Preparation of Artificial Gastric Fluid (AGF)

The artificial gastric fluid has been prepared on the basic gastric fluid and the pepsin. The basic gastric fluid has been prepared according to Clavel et al. (*12*) with some modifications. Briefly, 4.8g of NaCl (Wako Pure Chemicals, Osaka, Japan), 1.56g of $NaHCO_3$ (Wako Pure Chemicals, Osaka, Japan), 2.2g of KCl (Wako Pure Chemicals, Osaka, Japan), and 0.22g of $CaCl_2$ (Wako Pure Chemicals, Osaka, Japan) were dissolved in 1 L of distilled water. After the autoclaving at 121°C for 15 min, the pH of the basic gastric fluid was adjusted to 2.4 ±0.2 using 1 M HCl, and 2 mg of pepsin (Sigma Aldrich, USA) per 50 mL of the artificial gastric fluid was added and mixed well (AGF). AGF was stored at 4 ^{o}C while not in use.

Preparation of Artificial Chyme Emulsion (ACE)

The artificial diet was prepared by blending corn 11 g of oil, soybean lecithine 0.5g, corn starch 30g, casein powder 14 g, soy protein 5 g, pectin 2 g, mineral mix 2g, vitamin mix 0.5g, desiccated spinach powder 10 g with distilled water 25g thoroughly with a bench Blend YAP-214 (Taichung fine Machinery Co., Taichung, Taiwan) for

30 min and separately passed through the stainless sieve mesh #80, #60, #40, and #20 onto sterilized stainless pans, respectively. These screened particles were kept as loosely apart as possible in an aseptic room maintained at 4 ^{o}C (ACE).

Liquid Culture of H. pylori

The liquid culture medium was prepared according to Xia et al. (*13*) with a slight modification. Briefly, to 90 mL of brain heart infusion broth, 5 mL of horse serum, 0.25 mL of yeast extract, and. Vancomycin (50 mg) and amphotericin B (50 mg) were added. Double distilled water was added to make up 100 mL. The pH was adjusted to 6.5-7.0 with 1 M HCl. *H. pylori* ATCC 43579 was seeded at a density of 1×10^5 cells/mL and incubated at 37 ^{o}C under 5% CO_2 atmosphere for 48 h.

Preparation of H. pylori Contaminated Chymes

To ACE (80 g) 120 mL of ASL was added. Onto the semisolid chymes *H. pylori* was seeded at a density 2×10^5 bacteria cell/mL. The mimic chymes were aseptically pasted with a sterilized pestle for 5 min to mimic the chewing process in the mouth. The chyme mass was separately sieved with aseptic stainless screen to make particles with size #80, #60, #40, and #20 (particulate chyme groups, PCG).

Mimic Gastric Fluid Attack

To each 30 mL of AGF 2 g of original unscreened contaminated chyme mass, PCG of size #80, #60, #40, and #20 were respectively added to aseptic 50 mL reaction beakers.

The original unscreened contaminated chyme was used as the control. All other groups except the unscreened chyme mass were remained unstirred for 60 min, while the original unscreened contaminated chyme mass was intermittently stirred at 60 rpm for 1 min within every 5 min cycle (designated as the dispersed chime to mimic the homogeneous state of chymes in stomach). The total treatment time lasted for 60 min.

Enumeration of the Survival H pylori

Percent bacterial survival was analyzed by two experiments, one performed in a similar fashion as described in the section "Detection of *H. pylori* by Non-Invasive Stool Antigen Test". Starting from "The "chyme" antigen test was performed according to the manufacturer's recommendations using two different production lots……".. The other was performed by the plate cultivation described in the section "Plate Cultivation of *H. pylori*". The averaged data were taken.

Statistical Analysis

Data obtained in the same group were analyzed by Student *t* test with computer statistical software SPSS 10.0 (SPSS, Chicago, IL). Statistical analysis system ANOVA with Tukey test software was used to analyze the variances, and multiple range and test their significances of difference between paired means. A confidence level of $p<0.01$ was for the highly significant, and $p<0.05$ for the significant

difference. While a level of $p<0.20$ was used to evaluate the therapeutic effect before and after the treatment with FSL.

RESULTS AND DISCUSSION

The Proximate Composition

Fermentaion initiated the degradatrion and biotransformation of carbohydrates, ptoteins, and lipids. The composition of carbohydrates, proteins, and lipids decreased from 27.6 to 11.8g%, 32.2 to 12.4g%, and 25.4 to 11.5g%, respectively (Table 1). During fermentation, the environment becomes more suitable for the salt-tolerant organisms, such as *P. halophilu*s.

Isoflavonoid Content Increased Significantly During Fermentation

Microbial fermentation transformed many isoflavonoids, including genistin, daidzin, genstein, daidzein, glycitin and glycitein. The overall strength increased from 2.5 to17.3 folds. On the contrary, the content of malonylgenistin decreased significantly from 156.9 to 18.4 mg% (Table 2). During fermentation, 6-O-malonylglucosides are converted to 6-O-acetylglucosides or β-glucosides by heat treatment or action of β-glucosidases (*14*). A summary of cited nutritional and biochemical aspects of fermented soybean is shown in figure 1. Briefly, Yang et al. indicated that soy

isoflavone aglycones like genistein and daidzein are better absorbed than their corresponding glucosides (*15*) (Fig. 1). Soybean isoflavonoid and protein consumption can alleviate insulin resistance and glycemic control (*16,17*) (Fig. 1). Many soybean isoflavonoids exhibit estrogen-like structures (*18*). Genistein and daidzein bind weakly to receptor α but more strongly to receptor β, hence can exhibit organ-specific estrogenic and antiestrogenic effects (*19*) (Fig. 1).

Biological Effects of Soybean Protein, Peptides, and Amino Acids

Lunasin, a 43-amino-acid peptide from soybean, has anticancer and anti-inflammatory activities (*20,21*) (Fig. 1). The tripeptides (Val-Pro-Pro and Ile-Pro-Pro) act as antihypertensive agents in spontaneously hypertensive rats (*22*) (Fig. 1). The apparently increased contents of isoleucine, valine, and proline in FSL respectively from 1.0 to 3.8 mg%; from 0.8 to 3.2 mg%, and from 1.2 to 3.5 mg% implicated that the transformation of Val-Pro-Pro and Ile-Pro-Pro in FSL is very likely (Table 2) (*23*) (Fig. 1). We hypothesize that some ofl these above mentioned pharmacological effects can be pertinently pointing to the destruction of acidic microenvironment already built by action of proton pump (PP) device of *H. pylori* when they have successfully penetrated into the gastric mucosa (Fig. 1). In deed, soybean derived peptides currently have become the hot spot investigation for new drugs and functional food ingredients for gut health and modulating the intestinal absorption of nutrients.

Fermented Soybean Liquid Secured LDL Peroxidation.

Suppression of Conjugated Diene Formation

The formation of conjugated dienes was greatly reduced by FSL administration (Table 3). Only 3 μM remained in the FSL group at the end of experiment (Table 3). In LDL peroxidation, ox-LDL would first appear in the very early stage and easily protonated. The latter is then dehydrated to form dienes, among which conjugated dienes are the most stable (*3*).

Suppression of Conjugated TBARs Formation

As seen, FSL effectively suppressed the formation of TBARs. The FSL-treated showed a very low level of TBARs (38 μM) at the end of 3-month treatment (Table 3). In contrast, that of placebo consistently retained within 128-132 μM. In the early stage of LDL peroxidation, conjugated dienes usually first appears after a short period of lag time, followed by a serial subsequent oxidative process and then decomposed to produce a variety of decomposition products like aldehydes and ketones (*3*).

Na-Tou fungi, *Saccharomyces sp*. and *Asgergillus sp*. could produce a diversity of active antioxidative isoflavonoids through fermentation, all are good anti-lipid peroxidatives able to inhibit the oxidative modification of LDL by macrophages (*24*). Polyphenolics prevented ROS damage, like gastric hemorrhage, to human gastric epithelial cells *in vitro* and to rat gastric mucosa *in vivo* (*25*). Statistically, only

LDL-cholesterol can be significantly affected in the *H. pylori*-infected with greater disordered lipid metabolism (p>0.05) (*26*), implicating the potential therapeutic effect of FSL antioxidants on GPU.

Fermented Soybean Liquid Suppressed Growth of *H. pylori*

The resident number of *H. pylori* was attenuated by FSL-treatment. The *H. pylori* count in the placebo remained almost unaffected within 52-57 CFU/mL, while 82% of the patients were greatly improved by FSL treatment (Table 3). Thus, the viability of *H. pylori* in gastric mucosa can be a major determinant for prevalence of GPU. Tremendous clinical trials indicated that *H. pylori* eradication therapy can help gastric ulcer healing. Much of the literature has indicated that the pH value of environment is the main determinant affecting the survival of *H. pylori*. The viability of *H. pylori* is not affected within pH 5–7, however its growth and proliferation do not occur at pH ≤ 3 and ≥ 9, its flagella proteins are inactivated at pH ≤ 3.0. Below pH 6.0, *H pylori* organisms will survive but not divide (*27*). Previously a low-pH-inducible gene, *cagA* has been shown relevant to the survival and persistence of *H. pylori* in the gastric environment (*28*). *H. pylori* can survive in environment having pH within 3.0-9.0, but its growth and proliferation only can occur within pH 6-8, in fact the optimum pH range for its growth in gastric mucosa is 6.0-7.0 (*28*) (Fig. 2). Accumulating report elsewhere indicated that the *H. pylori* urease uniquely retards the acidic gastric fluid,

so that they can survive in gastric lumen. *H. pylori* produces high level HPU, which constitutes almost 6% of the soluble proteins) (*29*). The HPU secreted by *H. pylori* neutralizes gastric acid by decomposing urea to produce carbon dioxide and ammonia. The ammonia in turn neutralizes the gastric fluid to create a slightly acidic (pH 5.0-7.0) extracellular macroenvironment (Fig. 2) to accommodate a transient 'favorable' environment for penetration. Unlike that of other urease-positive bacteria being only localized in plasma, HPU simultaneously appears in cytoplasm and on cell surface (*29*,*30*). The HPU, a supramolecule composing of 12 copies of two subunits having molecular weight 61 kDa and 27 kDa respectively, coexists as $\alpha_{12}\beta_{12}$ with a low K_m value. Such a cluster of supramolecular assembly is crucial for the survival of the enzyme HPUre at low pH >5.0, at pH < 5.0, the enzyme is inactivated irreversibly (*31*). The question now arises how can *H. pylori* successfully stand the high strength gastric acid and successfully infect the gastric mucosa? And why FSL can effectively alleviate PU? It is really non-convincing that at the initial stage *H. pylori* could infect gastric mucosa which is in such a high acidic environment with pH 1.0-2.5.

Kinetic Analysis Reveals Bare *H. pylori* Unable to Directly Attack the Gastric Mucosa by Its Urease

Given in Table 4, the values of K_m and V_{max} of HPUre 0.21 ±0.06 mM (= 210 ±60 μM) and 1200 ±300 μM/min-mg (= 7.2×10^4 μM/h-mg), respectively (*32*), and the blood urea level S_{bu} =2.5×10^3 μM; the gastric fluid volume production rate (dV/dt)= 0.104 L/h at pH 2.0 (C = 0.01 M HCl) (*33*) (Table 4).

Since the blood urea level (S_{bu}) is far excess over the K_m value, HPUre always acts at its V_{max} provided that *H. pylori* cells have successfully colonized in gastric mucosa. In addition, the gastric fluid production rate R_{gf} is

$$R_{gf} = C(dV/dt) \quad \text{.....1}$$

$$= 0.01\ M \times 0.104\ L/h$$

$$= 1.04\times10^3\ \mu M/h \quad \text{.....2}$$

Accordingly, the amount of ammonia required to neutralize the gastric fluid at pH 2.0 to end point pH 7.0 for optimum growth of *H. pylori* should correspondingly match the same production rate 1.04×10^3 μM/h (Table 4).

Stoichiometrically, urease decomposes one mole urea to give 2 moles ammonia (Eq. 3).

$$NH_2\text{-}CO\text{-}NH_2 + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2 \quad \text{.....3}$$

For urea decomposition, the decomposition rate (R_u) in the gastric fluid macroenvironment only needs half the value of gastric fluid production rate (R_{gf})

$$R_u = R_{gf}/2$$

$$= (1.04\times10^3\ \mu M/h)/2$$

$$= 5.2\times10^2\ \mu M/h \quad \ldots\ldots 4$$

Apparently, to suppress the gastric acidity, the minimum amount of enzyme urease protein (W_{ure}) required would be

$$W_{ure} = R_u / V_{max}$$

$$[5.2\times10^2\ \mu M/h]\}/\ [7.2\times10^4\ \mu M/h\text{-}mg] \quad \ldots\ldots 5$$

$$= 7.2\times10^{-3}\ mg$$

$$= 7.2\times10^6\ pg \quad \ldots\ldots 6$$

As mentioned, the *H. pylori* urea protein constitutes almost 6% of the soluble proteins (30), which is approximately 0.06 *p*g/cell (Table 4), giving the urease protein of *H. pylori*

$$P_{urease} = 0.06\ pg/cell \times 0.06 = 3.6\times10^{-3}\ pg/cell \quad \ldots\ldots 7$$

Thus the initial colonization in the gastric mucosa at pH 7.0 requires a population of bare *H. pylori*

$N_{Hp} = 7.2\times10^{6}$ *p*g /3.6×10^{-3} *p*g/cell

$= 2.0\times10^{9}$ cells ……..8

Imagine that such a huge number of bare *H. pylori* cells should have been ready existing previously and sticking together immediately before colonization, a situation unlikely achievable in the extremely high strength acidic gastric lumen fluid.

As mentioned, *H. pylori* is survivable within pH 5–7, however unable to divide at pH ≤ 6.0. At pH≤ 3.0 or pH ≥ 9, its flagella proteins are completely inactivated (*27*). Considering the initial successful attack and attachment on the gastric mucosa would be the major determinant factor of *H. pylori* infection, we hypothesize herein the "Microenvironmental Chyme Protection Theory". Principally, there must be a "well buffered chyme shelter" protecting *H. pylori* to facilitate its initial attack onto the gastric mucosa. Only under such a circumstance, *H. pylori* can have the least chance to survive while HPU is likely acting as a fighter jet at the very early stage of colonization against the extremely tough extracellular macro-environment (here we designate it to be the infinitely small junction between the chyme and gastric mucosa surrounded by gastric fluid having pH 1.0-2.5 (Fig. 2). Conversely, PP acts as both a

519 protective and an invasive tool in the microenvironment once it has colonized a focus
520 in gastric mucosa (Fig. 2). To emphasize, the initial invasive power of *H. pylori* would
521 not depend on the neutralization power of HPU excreted by bare *H pylori* cells.
522 Instead, the flagella penetration and the neutralization of blood pH by action of PP are
523 playing the dominant attacking power. After penetrating into the gastric mucosa with
524 the help of energetic flagella, PP is more activated to sustain a slightly more acidic
525 microenvironment than blood within gastric mucosa to accommodate the survival and
526 proliferation of *H. pylori*. As mentioned, a pH range 6.0-7.0 is optimum for its growth
527 (*28*) (Fig. 2). Under such a condition with pH ≤ 7.0, a diversity of host immune
528 system simultaneously can be partially or completely suppressed. Moreover, the blood
529 pH is 7.25-7.45 (*33*). In essence, PP may multifunctionally act as an invasive factor, a
530 protector, as well as an immunity suppressor by attenuating and inactivating host
531 immune system by lowering the blood pH. Evidently, colonization of *H. pylori* in the
532 gastric mucosa needs a close coordination of both PP and HPU (Fig. 2). PP acidifies
533 whereas HPU alkalinizes the environmental fluid pH, operating as a well
534 compromized “Two-Point pH Modulator” and creating a focus maintained at pH 6.0
535 to 7.0. Supposedly, FSL intervenes and destroys such a well-controlled
536 microenvironment. To verify this hypothesis, we performed the Chyme-Shelter
537 Testing to simulate the infection path of *H. pylor*i. We prepared *H. pylori*-

contaminated chymes of different particle size having *H pylori* at a density of 2×10^5 bacteria cell/mL. The artificial gastric fluid with pH 2.4 ±0.2 and at 37 ^oC was used to mimic the gastric fluid attack on *H. pylori* during the infection. The sheltering effect was prominently perceived. The intact-, dispersed-, and particulate (mesh size #80, #60, #40, and #20) chymes in artificial gastric fluid showed the cell viability of HP to be time- and size-dependent (Table 5). Larger chyme particles showed more prevailing sheltering effect. For a chyme with a given particle size, the longer the contact time the larger population of *H. pylori* was killed. The cell viability was seen changing from 95.1% to 82.6%, 28.6% and 20.5 % respectively at time of contact from 0.0 to 20, 40 and 60 min (Table 5). While with the contact time fixed at 20 min, the viability was 100%, 4.2%, 31.4%, 43.3%, 57.2%, and 82.6% respectively for the intact-, dispersed-, and the particulate chymes with mesh size #80, #60, #40, and #20. Implicitly, at the moment of attack on the gastric mucosa, HP has to be first protected by chymes acting as a "sheltered HP cluster".

Obviously, the prevalence of GPU depends on a diversity of factors including intrinsic and extrinsic factors. The intrinsic factors involve the physiological status of each individual (e.g. gastric hyperacidity, the nutritional status, the internal stress, the immunostatus,…etc.), and the extrinsic factors involve the external stress, nutritional consumption, the source of *H. pylori*, the quantity of *H. pylori*, ……etc.) (Private

557 communication from the gastroenterologist, Prof. Pan, Taipei Medical University,
558 Taiwan). Based on these considerations, the nutraceutical and therapeutic role of FSL
559 is proposed in Table 6. In brief, the prerequisite requires that the host must have been
560 hyperacidic in stomach and simultaneously under big stress. Under such a
561 circumstance, the gastric mucosa will become thin enough to facilitate the penetration
562 of *H. pylori* hidden and protected in chymes (Table 6). At this stage, a minimum
563 critical population of *H. pylori* is required, and simultaneously, HPU is activated to
564 create a favorable macroenvironment having pH 5.0-7.0 (Table 6). After having
565 resided in the mucosa, PP of *H. pylori* is triggered to neutralize the alkaline blood
566 stream having a pH 7.25-7.45, maintaining the microenvironment at the optimum pH
567 6.0-7.0 for growth (Table 6). In brief, the effect of FSL to ameliorate PU can be very
568 diverse and extremely complicated depending upon the pathological condition.

569 In summary, fermentation increased the isoflavonoids and essential amino acid
570 contents by 2.5-17.3 and 1.5-4.0 folds in FSL. Kinetic analysis indicated that an
571 effective infection in gastric fluid having pH 2.4±0.20 requires a single cluster of
572 2.0×10^9 bare cells for a single one-strike attack, which is unlikely to occur in reality.
573 The Chyme-Shelter Testing showed the cell viability of HP was time- and
574 size-dependent. implicating the attack by a sheltered HP cluster is more likely. FSL
575 was shown beneficial to PU, 1g/20 mL t.i.d. eradicated HP in 82% of 37 volunteers

with PU after 3 month treatment. Simultaneously their plasma conjugated diene and TBARs contents were resumed ($p< 0.20$). Suggestively, the action mechanism of FSL may involve i) elevating blood alkalinity and buffering capacity to destroy the acidic microenvironment created by PP; ii) providing valuable bioactive anti-inflammatory and vasodilating peptides; iii) increasing blood flow to enhance nutritional supply to facilitate recovery of normal mucosa; iv) suppressing the ROS and interfering with macrophage recognition on ox-LDL.

Conclusively, kinetic analysis and the Chyme-Shelter Testing have revealed that the direct infection by bare *H. pylori* on the gastric mucosa is unlikely to occur in the real status. HP may sustain its growth and proliferation by “Two-Point pH Modulator”. Fermentation increases the isoflavonoids and essential amino acid contents. Administration of FSL resumes plasma conjugated diene and TBARs levels ($p< 0.05$). Thus, FSL is beneficial to eradicate the HP in GPU patients ($p<0.20$).

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Conflict of Interest

The authors do not have any conflict of interest in the submission of this work.

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Figure Legend

Fig. 1. Hypothesized pharmacological potential of fermented soybean liquid for treatment of gastropeptic uilcer.

Fig. 2. Role of urease and proton pump in colonization of *Helicobacter pylori* inside gastric mucosa.

Fig. 1. **Hui-Er Wang**

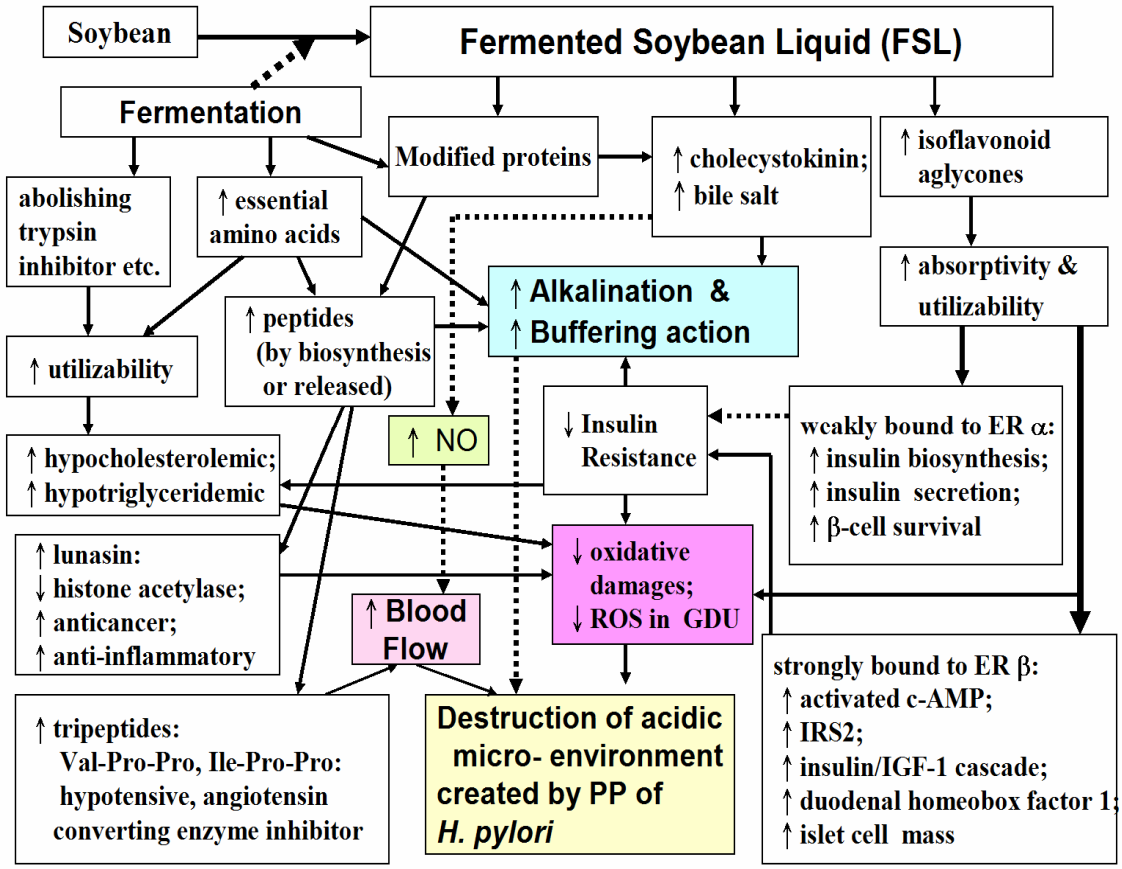
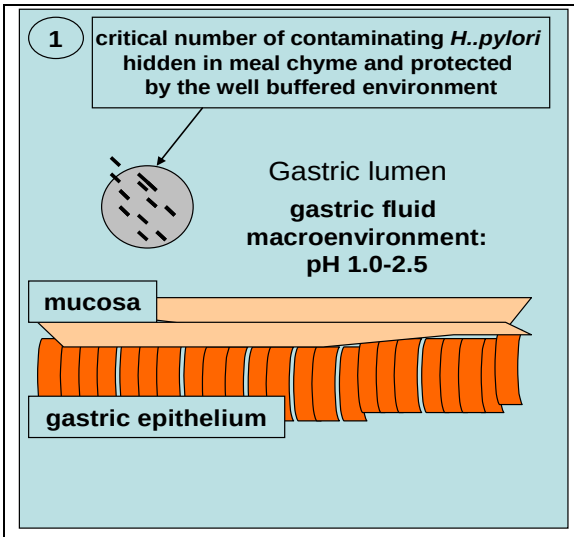
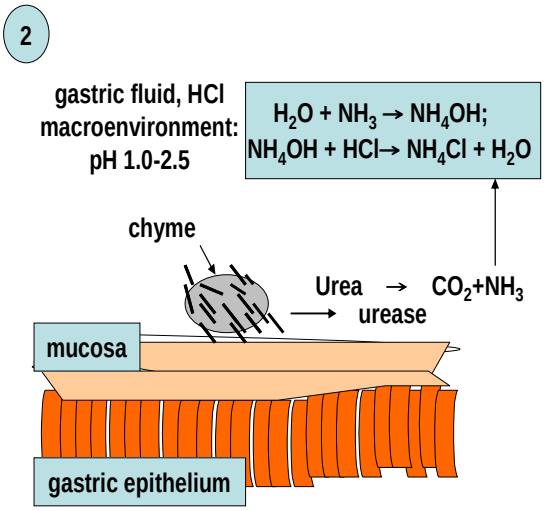
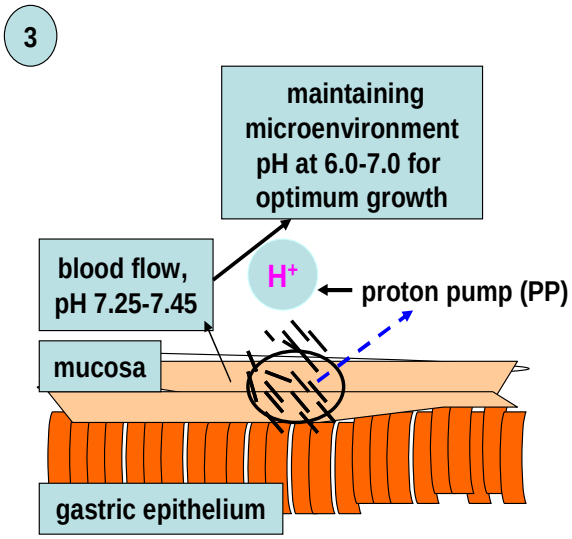


Fig. 2 **Hui-Er Wang**







Number 1: The gastric mucosa must have been thin enough due to long term hyperacid and stress, and the invading *H. pylori* must have reached a critical number hidden in the well-buffered chyme.

Number 2: On attaching to the mucosa, urease is triggered to maintain pH >6.0 in the transient penetrating hour.

Number 3: PP is triggered, pumping protons to neutralize blood pH and maintaining the microenvironment at pH <7.0 (optimum growth pH 6.0-7.0).

Table Caption

Table 1. Proximate compositional change prior to and post fermentation

Table 2. Change of isoflavonoid and amino acid contents in soybean products before and after fermentation.[a]

Table 3. Change of *H. pylori* count, and the LDL oxidative damages: TBARs and conjugated diene formation in LDL when treated with FSL.

Table 4. Parameters used to calculate the attacking of bare *H. pylori* on gastric mucosa from gastric lumen at pH 1.0-2.5.

Table 5. The percent viability of *H. pylori* in the artificial gastric fluid as a function of chyme particle size and contact time.[*]

Table 6. Hypothesized biological effect of FSL to eliminate *H. pylori in vivo* GPU victims.

Table 1. Proximate compositional change prior to and post fermentation.[a]

Item	Contents g%, (g/100g, dry basis)	
	Before fermentation	After fermentation
Carbohydrate	27.6±3.2[a]	11.8±2.3[b]
Protein	32.2±4.4[a]	12.4±2.2[b]
Lipid	25.4±3.5[a]	11.5±2.3[b]
Total isoflavonoids	154.8±22.5[a] (dry basis)	48.7±5.4 (wet basis) 556.7±26.8 (dry basis)[b]

[a]Data expressed in Mean±S.D. from triplicate samples.

Different superscripts of lower case in the same row indicate significantly different from each other (p<0.01).

821

822 **Table 2. Change of isoflavonoid and amino acid contents in soybean products**

823 **before and after fermentation.**[a]

compound	**content, mg%**		**compound**	**content, mg%**	
Isoflavonoids	**before**	**after**	**Amino acids**	**before**	**after**
genistin,	46.7±2.4	168.4±37.4	threonine	1.2±0.5	1.6±0.3
daidzin	35.8±3.2	105.6±17.4	cysteine	0.5±0.2	0.4±0.2
genstein	3.4±0.6	27.7±3.2	methionine	0.3±0.1	5.0±1.4
malonylgenistin	156.9±7.4	18.4±2.5	valine	0.8±0.2	3.2±0.7
daidzein	2.3±0.6	12.7±2.1	leucine	1.7±0.5	2.8±0.6
glycitin	3.3±0.7	10.2±3.1	isoleucine	1.0±0.3	3.8±1.1
glycitein	0.4±0.1	6.9±2.2	histidine	0.8±0.2	2.4±0.5
Amino acids					
aspartic acid	1.6±0.4	2.9±1.2	lysine	1.5±0.5	2.2±0.7
glutamic acid	4.3±1.3	7.9±1.7	phenylalanine	1.2±0.3	1.5±0.4
serine	1.2±0.3	1.8±0.8	tyrosine	1.0±0.4	2.3±0.6
arginine	1.7±0.4	1.9±0.7	proline	1.2±0.5	3.5±0.8
alanine	0.9±0.2	1.2±0.4			

824 [a]Data expressed in mean±S.D. from triplicate samples.

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Table 3. Change of *H. pylori* count, and the LDL oxidative damages: TBARs and conjugated diene formation in LDL when treated with FSL.[a]

Parameter	Duration of treatment (month)			
	0	1	2	3
H. pylori	-	-	-	-
(CFU/mL) (%)	-	-	-	-
Placebo	$56\pm9^{a,A}$ (100%)	$57\pm11^{a,B}$ (100%)	$54\pm8^{a,B}$ (100%)	$52\pm12^{a,B}$ (100%)
FSL	$57\pm10^{d,A}$ (100%)	$40\pm11^{c,A}$ (100%)	$12\pm3^{b,A}$ (23%)	$8\pm2^{a,A}$ (18%)
CD, μM[b]	-	-	-	-
Placebo	$25\pm9^{a,A}$ (100%)	$26\pm5^{a,B}$ (100%)	$24\pm8^{a,B}$ (100%)	$26\pm4^{a,B}$ (100%)
FSL	$25\pm9^{d,A}$	$12\pm4\ 9^{c,A}$	$5\pm2^{b,A}$	$3\pm2^{a,A}$ (100%)
TBARs, μM[b]	-	-	-	-
Placebo	$128\pm49^{a,A}$ (100%)	$134\pm36^{a,B}$ (100%)	$130\pm44^{a,B}$ (100%)	$132\pm36^{a,B}$ (100%)
FSL	$132\pm33^{d,A}$	$112\pm25^{c,A}$	$58\pm11^{b,A}$	$38\pm8^{a,A}$ (84%)

[a]Samples of LDL (125 μg protein/mL) were obtained at month 0, 1, 2, 3 respectively from the placebo and the FSL treated groups. The superscripts in upper case designates significant difference between the placebo and the FSL-treated parameter in each column. The superscripts in lower case show significant time dependent differences among data in each row.

[b]The reference molar absorptivity of TBARs at 532 nm $\varepsilon_{532} = 1.56\times10^5\ M^{-1}\ cm^{-1}$ (Hsieh et al., 2005). The reference molar absorptivity of conjugated diene (CD) at 234 nm is $\varepsilon_{234} = 2.95\times10^4\ M^{-1}\ cm^{-1}$) (Hsieh et al., 2005).

853

854 **Table 4. Parameters used to calculate the attacking of bare *H. pylori* on gastric**
855 **mucosa from gastric lumen at pH 1.0-2.5.**

Parameters	Cited value	Value used for calculation	Reference
Gastric production rate	2500 mL/24 h; or 104 mL/h; or 0.104 L/h	0.104 L/h	Harper et al., 1977
pH of gastric fluid	1.0-2.5	2.0 = 0.01 M HCl	Harper et al., 1977
K_m of urease	0.21± 0.06 mM	0.21 mM; or 210 μM	Gang et al., 2009
V_{max} of urease	1200 ± 300 μM/min-mg	7.2×10^4 μM/h-mg	Gang et al., 2009
Blood urea nitrogen (BUN)	8-20 mg/dL; or 2.86-7.14 mmol/L	140 mg/L; or 5 mM	Harper et al., 1977
Blood urea	(taking the median value from BUN to calculate)	600 mg/L; or or 2.5×10^3 μM	Harper et al., 1977
E. coli cell size volume wet mass dry wet mass, DCM cell count total protein soluble protein	- ℓ= 2 μm, w = 0.5 μm; 0.6-0.7 μm^3 ≈ 1 *p*g; ≈ 0.2 *p*g DCM 10^9 cells/g wet mass assume= 40%DCM (0.08 *p*g) assume = 15%DCM (0.03 *p*g)	- - - - - - - -	Wikipedia
H. pylori size volume wet mass dry wet mass, DCM cell count total protein soluble protein	- ℓ= 3-4 μm, w = 0.5-1 μm; 1.2-1.4 μm^3 ≈ 2 *p*g; ≈ 0.4 *p*g DCM 5×10^8 cells/g wet mass assume= 40%DCM (0.16 *p*g) assume= 15%DCM (0.06 *p*g)	- ℓ= 4, w = 0.5 - - - - - 15% (0.06 *p*g)/cell	- - Calculated taking E. coli as the reference data base

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Table 5. The percent viability of *H. pylori* in the artificial gastric fluid as a function of chyme particle size and contact time.[*]

Contact time (min)	Particle size of chymes/ % viability					
	intact chyme	dispersed chyme	#80 mesh	#60 mesh	#40 mesh	#20 mesh
0	$100\pm0.1^{c,C}$	$92.3\pm3.1^{a,B}$	$92.5\pm2.3^{a,C}$	$92.9\pm2.5^{a,C}$	$93.4\pm2.3^{a,D}$	$95.1\pm3.1^{b,D}$
20	$100\pm0.1^{f,C}$	$4.2\pm1.5^{a,A}$	$31.4\pm2.1^{b,B}$	$43.3\pm3.6^{c,B}$	$57.2\pm5.2^{d,C}$	$82.6\pm4.2^{e,C}$
40	$96.2\pm2.2^{e,B}$	0.0	$14.5\pm2.2^{a,A}$	$18.5\pm2.4^{b,A}$	$21.3\pm3.3^{c,B}$	$28.6\pm3.2^{d,B}$
60	$92.3\pm1.8^{c,A}$	0.0	0.0	0.0	$5.3\pm4.2^{a,A}$	$20.5\pm4.2^{b,A}$

[*]The artificial gastric fluid (AGF) was 1 M HCl (pH 2.4 ±0.2) containing 4 mg of pepsin (Sigma Aldrich, USA) per 100 mL AGF. Data are expressed as Mean ± SD obtained from triplicate samples (n =3). Different superscripts of lower case in the same row indicate significantly different from each other ($p<0.05$ or $p<0.01$). Different superscripts of upper case in the same column indicate significantly different from each other ($p<0.05$ or $p<0.01$).

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879 **Table 6. Hypothesized biological effect of FSL to eliminate *H. pylori in vivo***

880 **GPU victims.**

Stage	**Gastric status**	***H. pylori* (HP) status**	**Host status**	**Role of FSL**
Normal uninfected stage	normal acidity (pH 1.0-2.5), normal mucosal thickness and parietal cell population.	although contaminated in foods, incapable to penetrate the gastric mucosa. (optimum survival pH range 5.0-8.0)	normal gastric physiology and biochemistry (blood pH 7.25-7.45; gastric pH 1.0-2.5)	nutrition supplement, hypoglyceridemic, hypocholesterolemic, antioxidant
Pre-infection stage	hyperacid, loss of parietal cells and mucous cells; thinner mucosa	a minimum critical population of HP hidden in a well emulsified and buffered chyme.	high stress both physically and mentally, malnutrition due to insufficient protein uptake	nutrition supplement, hypoglyceridemic, hypocholesterolemic, antioxidant
Initial infection stage	hyperacid, very thin mucosal lining.	HP in chyme attaches to mucosal surface, triggering urease (URE) to retard the acidic macro environment nearby mucosa. On penetrating into mucosa, proton pump (PP) is activated to neutralize blood pH and create an acidic micro environment.	appetite loss, nausea, simultaneously, host immune system is suppressed.	nutrition supplement, buffering to retard the acidification of microenvironment created by small pioneer population of HP; triggering host immune system; anti-inflammatory
Colonization stage	persistent gastric inflammation	colonized in mucosa, destroying parietal and mucous cell with aid of PP; migrating to gastric epithelium by help of PP with pH maintained at 5.0-7.0	stomach ache, nausea, inflammation, no appetite, dyspepsia	buffering to retard the acidification of microenvironment of medium population of HP, activating host immune system, anti-inflammatory,
Epithelial invading stage	epithelial erosion; gastric hemorrhage,	invading into gastric epithelial macro-environment with the help of PP and URE to maintain pH at 6.0-7.0 for optimum growth	inflammation, dyspepsia, gastritis, malnutrition, dystrophia, dyspepsodynia	buffering to destroy acidic micro-environment of huge population of HP; anti-inflammatory; lowing ROS and stress.
Gastro-peptic ulcer (GPU) stage	gastric hemorrhage, gastroalbumino orrhea	huge population existing in stomach, causing stomach erosion and ulcer; breath test positive.	ulcer, nausea; ammonia breath	buffering; lowering ROS and stress, anti-inflammatory

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