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Medical laboratory Department

Under title

Effective role of probiotic as supplementation cultured by special media in protection from H.pylori

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﴿ نَرْفَعُ دَرَجَاتٍ مَّن نَّشَاءُ وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ ﴾

صدق الله العلي العظيم

سورة يوسف (الآية:76)

Dedication

إلى من بلغ الرسالة وأدى الأمانة .. ونصح الأمة ..

إلى نبي الرحمة ونور سيدنا محمد صلى الله عليه واله وسلم ونهدي عملنا المتواضع هذا الى المخلصين من أبناء هذا البلد (شهدائنا الأبرار) الذين ضحوا بدمهم من اجل أن يرتقي العراق ويبقي عزيزاً ونعاهدهم على أن نكون أوفياء لهم من خلال أكمال المسيرة التعليمية سائلين المولى عز وجل أن يتقدمهم برحمته وغفرانه والى من كان سند لنا في هذه الدنيا وبفضل جهودهم وصلنا إلى ما نحن عليه ألْان .

(الوالدين) .... الى أخواني وأخواتي ...

الى الزملاء والأصدقاء الذين واصلوا معنا المشوار الدراسي والى الهيئة التدريسية

في

المعهد التقني المسيب / قسم تقنيات المختبرات الطبية

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وفٌي مقدمتهم أستاذي المشرف على الرسالة فضٌيلة **الأستاذ الدكتور / احمد صادق جاسم**

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رئٌس قسم ا تقنيات المختبرات الطبية وفقهما لكل خٌير لماٌ يبذلانه من
اهتمام بطلاب المعهد التقنٌي المسيب بصفة عامة
وطلاب المختبرات الطبية بصفه خاصة

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Chapter One

Introduction

Helicobacter pylori is a well-known pathogen that is highly prevalent in the world population, and *H. pylori* infection is potentially hazardous to humans because of its relationship to various gastrointestinal diseases, such as gastric ulcers, duodenal ulcer, chronic gastritis, and gastric carcinoma. Therefore, the clinical guidelines recommend taking antibiotic therapy to eradicate the pathogen, which usually leads to the desired therapeutic effect (Ji, J., & Yang, H, 2020). However, some failure cases of this therapy indicate that the increasing antibiotic resistance and side effects may affect the therapeutic effect. Here we propose that using probiotics as supplementation for antibiotic therapy may provide an extra help. Recent studies have shown that probiotic supplementation therapy has promising application prospects; it can enhance the antibiotic effect to achieve a better therapeutic result and maintain the balance of the host gastrointestinal microbiota. In summary, under global conditions of increasing *H. pylori* prevalence, probiotic supplementation therapy is worthy of further studies for future clinical application (Ji, J., & Yang, H. 2020, Nasution *et al*., 2020).

This study Has been suggested and designed to fulfill the following objectives :-

1- Production of beneficial bacteria in a selective culture medium to enhance the immunity of the digestive system.

2- Testing the ability of beneficial bacteria to suppress or stop the pathogenesis of harmful bacteria.

3- Pathological study of H.pylori bacteria by dosing it in laboratory animals to know the tissue damage.

4- Testing the probiotics by administering them in laboratory animals to know their effectiveness in protecting against different microbes .

Chapter Two

2.1 GENERAL DESCRIPTION

2.1.1 Microbiology

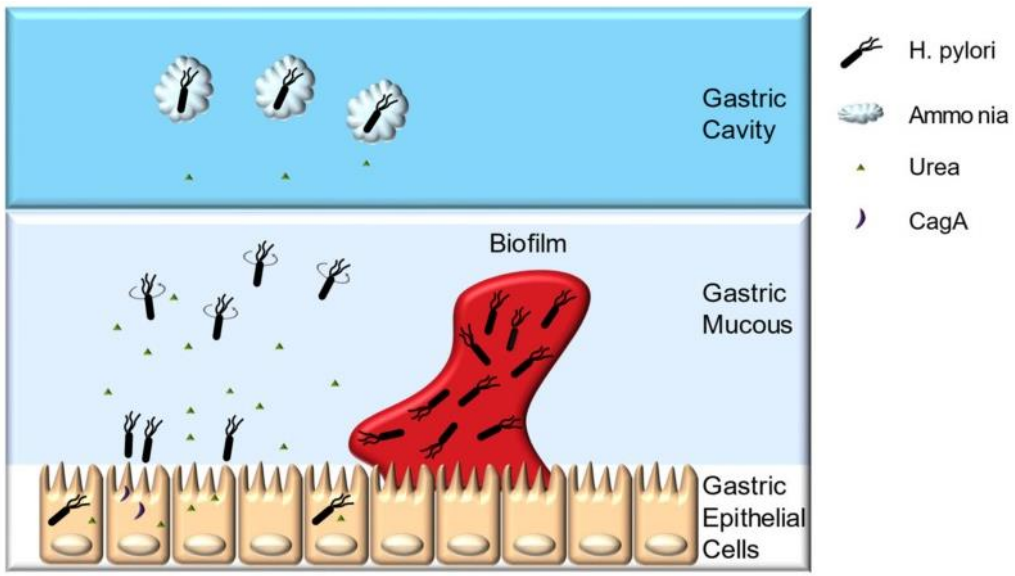
Helicobacter pylori is a microaerobic, spiral, flagellated Gram-negative pathogen that has colonized approximately 50% of the world's population yet, the infection rate in China has exceeded 80% and may continue to increase in the future **(Nasution *et al*., 2020, Tanabe *et al*., 2018).**Once *H. pylori* successfully colonized the stomach, it evolves toward persistent chronic infection with spontaneous clearance being relatively rare (Pacifico *et al*., 2014). Although the majority of infected individuals are clinically asymptomatic, the host can develop gastric ulcers, chronic gastritis or other gastrointestinal diseases, 1–3% of *H. pylori*-infected people are at risk of developing gastric cancer**(Pacifico *et al*., 2014).** Thus, medical guidelines recommend antibiotic therapy as a good option for clinical eradication of *H. pylori***(Pacifico *et al*., 2014)**. Recent studies have shown that the eradication of *H. pylori* in infected asymptomatic individuals at all ages can reduce the occurrence of gastric cancer **(Bae *et al*.,2018).** However, failure

cases in this antibiotic therapy indicate that drug-resistant strains and side effects may occur in some patients, which can affect the treatment effect**(Dang *et* al ., 2014).**

H. pylori can cause diseases only if successfully colonized. The optimal growth pH for *H. pylori* is 8.5, whereas the bacteria can survive for only approximately 30 min under extremely acidic environments, such as gastric cavity**. (Schreiber *et al* , .2005.Liu *et al* ,.2018)**

The ability of *H. pylori* to transiently resist gastric acid and pass through the gastric mucous layer, quickly reaching the pH-neutral environment, mainly depends on its urease, chemotaxis system, flagella, and spiral morphology

Figure 1



Colonization of the stomach by *H. pylori*.

Figure1 (2-1) Colonization of the Stomach By H. Pylori(**Salama *et al* ,.2013)**

In addition, the capacity for gastric epithelial cell adherence, biofilm formation, and antioxidant enzyme system help *H. pylori* achieve long-term colonization.(**Hathroubi *et al.*,2018 .Yonezawa ,*et al* 2015 . Yonezawa.et al., 2019)***H. pylori*** urease accounts for approximately 10% of its total protein mass,

playing a pivotal role in both establishing initial colonization and maintaining chronic infection **(Keilberg et*al*.,2016)**Urease can hydrolyze urea to produce carbon dioxide and ammonia, and the latter being able to buffer the gastric acids around the bacteria to maintain its viability. Furthermore, the morphology of the gastric mucin is closely related to the pH value. Gastric mucin forms a gel under low pH, whereas the increase in pH caused by urease catalysis loosens gastric mucin, enabling *H. pylori* to swim more easily**.(Debowski et *al.*,2017)**

The chemotaxis system, flagella, and spiral morphology of *H. pylori* enable its swift passage through the gastric mucous layer. Urea is not only the substrate of urease, but also one of the signaling molecules of this chemotaxis system. *H. pylori* uses chemotaxis system to sense the pH gradient, urea and amino acids secreted by the host cells to position itself **(Huang *etal*.,2015)**

The flagella and spiral morphology of *H. pylori* facilitate its passage through the gastric mucous layer. *H. pylori* has polar flagella with rotation that is powered by the motor proteins MotA and MotB, and mutants with incomplete flagella has a reduced ability to infect mice gastric, whereas mutants with more flagella number can swim faster through simulated gastric mucous layer. Spiral morphology enables *H. pylori* to drill through the gastric mucous layer like a rotating cork, mutants with straight-rod morphology will lose about 7–21% of its swimming speed **(Martínez *et al*.,2016)**

2.1.2. H. pylori Pathogenesis

Although about 85% of colonized individuals are asymptomatic or have mild gastritis, 15% of infected people still have a chance to develop peptic ulcer disease (PUD) during the long-term *H. pylori* infection, and about 1% can develop gastric

cancers. After successful colonization of the stomach, these bacteria can activate host's innate and adaptive immune response, which may induce atrophic gastritis, dysplasia, metaplasia, and ultimately gastric carcinoma. Recent studies have shown that the eradication of *H. pylori* in infected asymptomatic individuals at all ages can reduce the occurrence of gastric cancers. Various virulence factors and virulence genes of *H. pylori*, such as cytotoxin-associated gene A protein (CagA), vacuolating cytotoxin protein (VacA), duodenal ulcer promoting gene (DupA), and urease play the key role in injuring host tissues and inducing gastrointestinal diseases**.(Chang *et al.*,2018. Qureshie*tal*.,2019)**In addition, *H. pylori* urease and its catalytic products may cause direct damage to host tissues. Ammonium ions can destroy the integrity of the connection between gastric epithelial cells, while carbon dioxide supports bacteria resistance to damage from nitric oxide metabolites and peroxynitrite produced by phagocytic cells**. (Debowski *et al.*,2017)**

Urease also induces inflammation and angiogenesis in vivo independently of its catalytic activity and directly activates human neutrophils to produce reactive oxygen species, thereby injuring the host body**.(de Jesus Souza *et al* .,2019)**

2.2. PROBIOTIC THERAPY

2.2.1. Antagonistic Mechanism

As an emerging adjuvant, probiotics have been used to treat a series of gastrointestinal diseases, including *H. pylori* infection. In vitro experiments demonstrated that various probiotics have the potential to antagonize *H. pylori* through their metabolites or bacterial cells**. (Sun *et al* .,2018. Zhao*et al* .,2018)**

Sun et al. found four *Lactobacillus* strains isolated from fermented foods in northeastern China were able to inhibit the growth of *H. pylori*.**(Sun*et al*.,2018)**

The acid-resistant strain *L. johnsonii* No.1088, isolated from gastric juice of healthy volunteers could suppress *H. pylori* both in vitro and in a mouse model, and the heat-killed form of the strain also showed antibacterial effects **(Aiba*etal*.,2015. Aibaet al .,2017)**

The antagonism of probiotics against *H. pylori* is achieved through a series of direct or indirect interactions, including secreting antibacterial substances, competing inhibition, enhancing mucous barriers, and regulating immunity

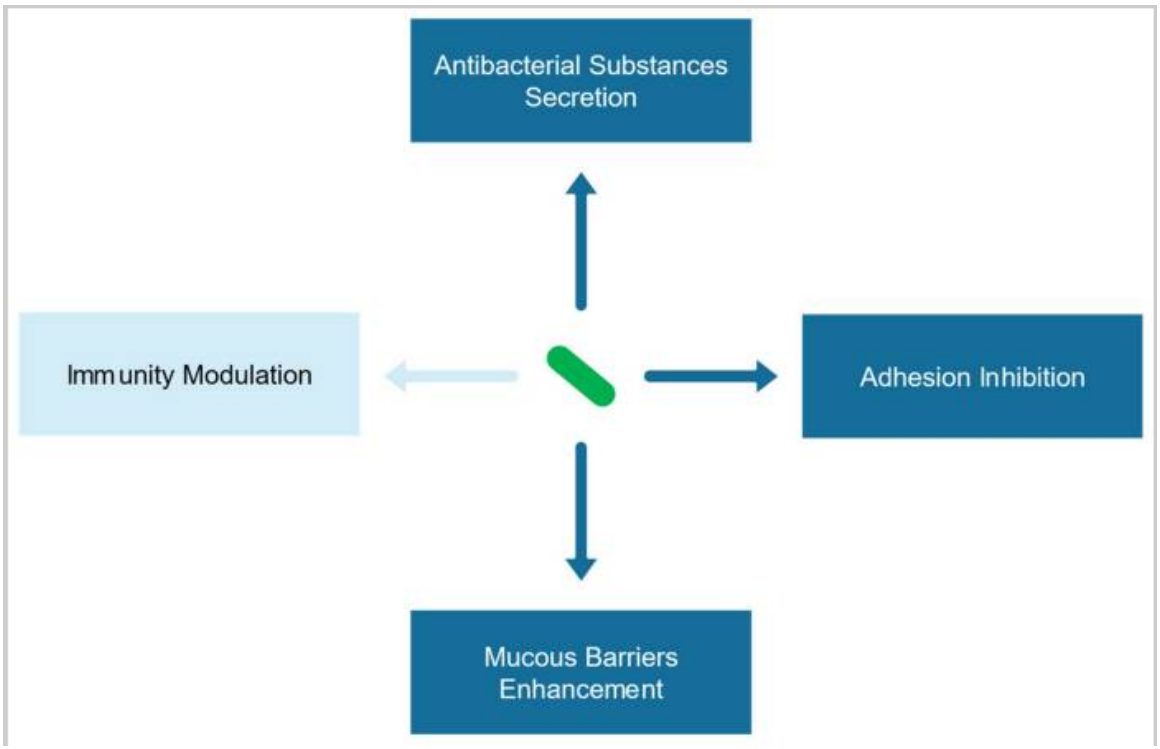


Figure2 (2-2) Antagonistic Mechanism of Probiotic Against H. Pylori (Qureshi e*t al*., 2019)

Probiotics can secrete antibacterial substances such as lactic acid, short-chain fatty acids (SCFAs), hydrogen peroxide, and bact**(Homan & Orel .,2015)** Lactic acid and short-chain fatty acids normally show more intensive antibacterial ability than

strong acids because of their incomplete ionization, the undissociated form of these organic acids can damage *H. pylori* cells by functioning as proton carriers that induce acidification of the cytoplasm and the accumulation of toxic anions.**(Jackson*et al.*, 2015)** Zheng et al. conducted an in vitro study on *L. pentosus* LPS16, and found that lactic acid can inhibit both drug-sensitive and drug-resistant *H. pylori* strains.**(Zheng*etal*., 2016)** Meanwhile, lactic acid can suppress *H. pylori* urease activity **(Lesbros-Pantoflickova D *et al*., 2007)** In addition to organic acids, hydrogen peroxide produced by probiotics can cause oxidative damage to pathogenic proteins, membrane lipids and DNA by forming peroxygen ions, thus injuring the *H. pylori* cell.**(Batdorj B*et al*.,2007)**Furthermore, certain probiotics can produce bacteriocins that have a direct antibacterial effect on *H. pylori*. Most bacteriocins are thermostable peptides with antagonistic activity against planktonic cells and/or biofilm cells.**(Kim N.-N*etal*.,2019)** Among seven bacteriocins derived from lactic acid bacteria, lacticin A164 and BH5 secreted by *Lactococcus lactis* showed the greatest effectiveness against *H. pylori* ATCC43504 and DSM.**(Kim T.S*et al* ., 2003)**Boyanova et al. found that the bacteriocin secreted by seven *L. bulgaricus* strains not only inhibited the growth of antibiotic-sensitive *H. pylori* strains, but also the antibiotic-resistant strains **(Boyanova L*et al*., 2017)**In addition to these peptide-like bacteriocins, a nonpeptide antipathogen substance synthesized by *L. reuteri*, called reuterin, can inhibit *H. pylori* growth and downregulate the expression of the virulence genes *vacA* and *flaA*.**(Urrutia-Baca V.H*et al* .,2018)**Probiotics may hinder *H. pylori* colonization by competing for binding sites or disturbing the adhesion process. Probiotics with high affinity for epithelial cells can block the colonization of pathogenic bacteria in gastrointestinal epithelial cells. It has been shown that *L. reuteri* JCM1081, TM105 can compete with *H. pylori* for the asialo-GM1 and sulfatide binding sites in gastric epithelial cells, thereby inhibiting early *H. pylori* colonization.**(Mukai T*et al* ., 2002)**Two *L. gasseri* strains can affect *H. pylori* colonization by inhibiting the expression of *H. pylori* adhesion gene *sabA*.**(De**

Klerk N *et al* ., 2016)*Saccharomyces boulardii* has neuraminidase activity selective for α(2,3)-linked sialic acid of host cell, thus removes *H. pylori* binding sites. **(Sakarya S&Gunay N ., 2014)**The enhancement of the mucous barrier by probiotics helps the host to hinder *H. pylori* colonization. *H. pylori* infection can downregulate the expression of *muc1* and *muc5AC* gene in KATO III cells, which may cause mucous layer disruption in vivo.(**Byrd J.C *et al* .,2000)**Probiotics can upregulate tight-junction proteins and promote the mucous secretion by increasing the expression of *muc1*, *muc2*, and *muc3*, thus stabilizing the mucous layer. **(Suez J *et al* .,2019)**These properties indicate that the host body may better resist *H. pylori* invasion by relying probiotics on repairing the gastric mucosal barrier, effectively preventing the initial infection and reinfection of the pathogen**. (Ji*et al* .,2020)**

2.2.2 Enhancement of the mucous barrier by probiotics

The enhancement of the mucous barrier by probiotics helps the host to hinder *H. pylori* colonization. *H. pylori* infection can downregulate the expression of *muc1* and *muc5AC* gene in KATO III cells, which may cause mucous layer disruption in vivo.(**Byrd J.C *et al* ., 2000)** Probiotics can upregulate tight-junction proteins and promote the mucous secretion by increasing the expression of *muc1*, *muc2*, and *muc3*, thus stabilizing the mucous layer.These properties indicate that the host body may better resist *H. pylori* invasion by relying probiotics on repairing the gastric mucosal barrier, effectively preventing the initial infection and reinfection of the pathogen. **(Suez J *et al* ., 2019)**In addition to these nonimmune effects, probiotics can alleviate the host inflammation caused by *H. pylori* infection. *H. pylori* infection-induced inflammatory diseases are associated with the sustained expression of inflammatory factors, and these factors do not eliminate *H. pylori* but

lead to the continuation of the inflammatory response.**(Yang Y.-J *et al* ., 2012)** Probiotics can inhibit pro-inflammatory factor expression, thereby mitigating the inflammatory response. Numerous studies have shown that probiotic strains such as *L. acidophilus*, *L. bulgaricus*, and *L. rhamnosus* can reduce the expression of IL-8 in *H. pylori*-infected cells.**(Quigley E.M.M.,2019,Chen Y.H.*et al* ., 2019)**Yang et al. demonstrated that although *H. pylori* infection causes the overexpression of IL-8, TNF-α and other pro-inflammatory factors in MKN45 cells, pretreatment with high doses of *L. acidophilus* La5 can silence the Smad7 and NF-κB pathways, thus relieving the inflammatory respons **(Yang Y.-J *et al* ., 2012)** Therefore, probiotics have a preventive and mitigating effect on the inflammation caused by *H. pylori* infection.**(Ji *et al* .,2020)**

2.3. Advantages of Probiotic Supplementation Therapy

2.3.1. Drug Synergy and Mutant Prevention Theories

The range of drug concentrations for which drug-resistant mutants are most readily induced is called the mutant selection window and it extends from the minimum inhibitory concentration (MIC) to the mutant prevention concentration (MPC). (**Marcusson L.L*et al* .,2005)** When the drug concentration is within the mutant selection window, the growth of drug-sensitive strains is inhibited, whereas the proportion of resistant strains iincreases. (**Feng Z.-Het al .,2019)** Although taking a low dose of drugs does not lead to a high incidence of resistant strains, it does not achieve good clinical effects, and increasing the dose might eradicate the pathogen with aggravating side effects. Multiple drug combinations can better resolve these issues.**(Brooks B.D &Brooks A.E. ,2014)**According to drug synergy theory, multiple drugs used together do not yield a simple effect of 1 + 1 = 2; they may have a synergistic or antagonistic effect. **(Caesar L.K&Cech**

N.B.،2019) Synergistic effects can improve drug efficacy and prevent the emergence of drug-resistant bacteria because of the difficulty in producing multiple drug mutants**. (Jia J*et al* .,2009)** Thus, using synergistic drugs for *H. pylori* treatment can decrease MPC and minimize the mutant selection window to achieve better efficacy and prevent an increase in drug-resistant bacteria. The synergistic effect is not limited to antibiotics, antibiotics and non-antibiotic adjuvants also have synergistic effects**. (Brooks B.D. & Brooks A.E ،. 2014)**Although most probiotics are localized in the gut, certain probiotics can colonize the pH-neural part of the stomach. (**Ryan K.A*et al.,* 2008)**Substances metabolized by these strains may have the potential to act synergistically with antibiotics. In vitro experiments have shown that the combination of tetracycline and probiotic fermentation broth has greater antimicrobial effects against *Pseudomonas aeruginosa* clinical resistant strains than either tetracycline or probiotic fermentation broth does alone.**(Soleymanzadeh Moghadam S et al .,2018)**Yang et al. conducted an antibacterial study on *Clostridium difficile*, and the results showed that *Bifidobacterium breve* YH68 cell-free supernatant can enhance the synergistic effect of antibiotics and weaken the antagonistic effect. **(Yang J&Yang H.,2018)**Consequently, a combination of probiotic metabolites and antibiotics can have a greater antibacterial effect against either Gram-negative or Gram-positive pathogens. Although there are a few in vitro studies on *H. pylori* to confirm the synergy between probiotics and antibiotics, the positive clinical results hint at a potential interaction**. (Ji, J & Yang, H.,2020)**

2.3.2. Gastrointestinal Theory

The human gastrointestinal microbiota theory suggests that gastrointestinal microbes and their metabolites can modulate human physiological activities, such as nutrient absorption, energy metabolism and immune function, and the bacteria,

host, and environment are interdependent and mutually constrained in a dynamic balance**. (Nicholson J.K *et al* .,2012)***H. pylori* infection alters both the gastric and intestinal microflora.**(Kienesberger S *et al* ., 2016)** At the same time, the bactericidal effect of antibiotic use and the pH change caused by PPI can lead to more severe disorders.**(Kienesberger S*et al* ., 2016)**Clinical studies have confirmed that *H. pylori* infection reduces the diversity of the stomach microbiota in children and adults, and this microbiota may not be restored after eradicating the pathogen.**(Shin C.Met al .,2019)** The evidence from an analysis of the gastric microbiota of infected people and noninfected people revealed that the abundance of *Proteobacteria* and *Spirochetes* in the former is higher than that of the latter**. (Shin C.M*et al* .,2019)** In mouse model, *H. pylori* infection changes the intestinal relative microbial abundance in an indirect way based on the fact that the bacteria has not been detected in mice feces.**(Kienesberger S*et al* .,2016)** Furthermore, antibiotic eradication therapy create additional changes in the gastrointestinal microbiota. *Lactobacillus* spp.in the stomach dramatically decreased after penicillin was administered to a mouse model. **(Espinoza J.L*et al* ., 2017)**In an infected population treated with quadruple therapy, the alpha diversity and *B. adolescentis* abundance in the gut microbiota was significantly decreased, and some *E. faecalis* strains acquired even greater antibiotic resistance**. (Olekhnovich E.I *et al* .,2019)**Intake of probiotics may produce positive effects in terms of protecting and recovering the gastrointestinal microbiota**(Gareau M.G *et al* ., 2010)**Experiments with animal models and clinical trials have shown positive effects of probiotics on the host gastrointestinal microbiota. After treatment with *L. rhamnosus* GMNL-74 or *L. acidophilus* GMNL-185, the abundance of *Bifidobacterium* spp. and *Akkermansia muciniphilia* in *H. pylori*-infected mice was significantly increased**. (Chen Y.H*et al* ., 2019)**Wu et al. found that the diversity of the gut microbiota is remarkably reduced when *H. pylori* infected individuals are treated with triple therapy alone, while supplementation with *Bacillus subtilis* and *E. faecalis* can inhibit this reduction.**(.Wu L*et al* ،.2019)**Moreover,

colonization of specific probiotics in gastric likely maintain the balance of the gastric microbiota**.(Espinoza J.L*et al* ., 2018)**Therefore, probiotic supplementation is effective for maintaining both the gastric and gut microflora during *H. pylori* infection and antibiotic therapy. **(Ji, J., & Yang, H.,2020).**

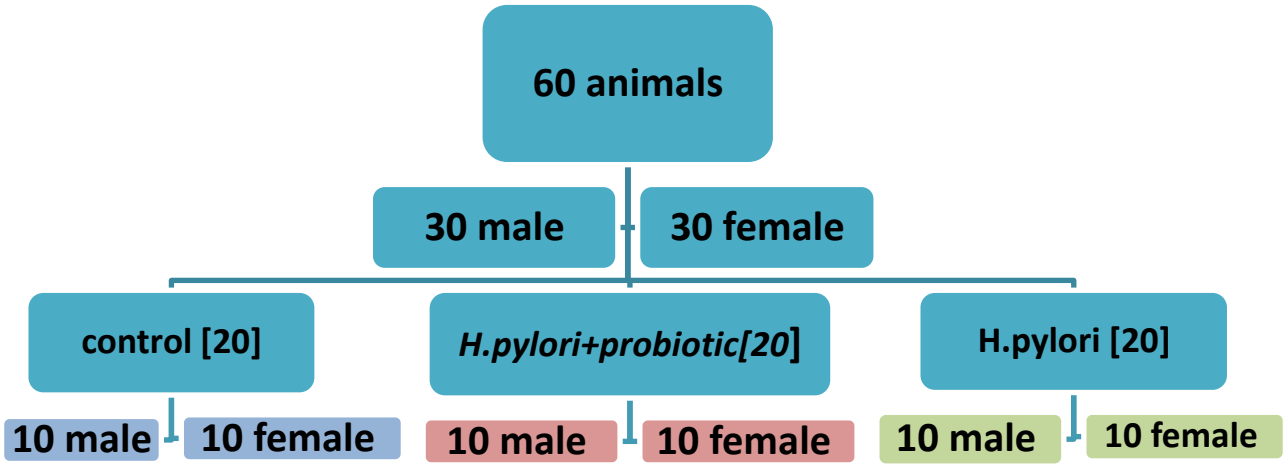
Chapter Three

3.1 Animals: Specifications of laboratory animals (rabbits) in this experiment :-The number of animals is (60 laboratory animals) divided into three groups, where the number of each group (20 laboratory animals) includes (10 males and 10 females).The weight of the first group is both females and males (0.5-1 (kg).The weight of the second group for each of the females and males (1-1.5 kg The weight of the third group for each of the females and males (1.5-2 kg)**.**

3.1.1 Sample: Stool sample and Blood sample , We collected the samples from 50 people infected H. Pylori the age about 18_50 years old .25 stool,,25 blood sample .

Design the Distribution of animals with H.pylori

Table (3-1)



3.1.2 Reagent and Chemical Solution Materalas

The reagent and chemical solution materals used in this work are shown in Table (3-2).

Table (3-2) Reagent and Chemical Solution

NO.	Material	Company\Country
1.	Nutrient agar	Hi_Media India
2.	Blood agar	Hi_Media India
3.	The Broth	China

4.	The Stain	Olympus
5.	Formalin 10%	Germany
6.	Catalase test	Korea

3.1.3 Laboratory Instruments:

The instruments used throughout this study are listed in Table (3-3).

Table (3-3) Laboratory Instruments and Equipment

NO.	Instrument	Company/Country
1.	Light Microscope	Korea
2.	Balance	Memmert, China
3.	Autoclave	Meheco(China)
4.	Incubator	China
5.	Refrigerator	Japan
6.	biosafety Cabinet(hood)	Olympus/Japan
7.	Disposable Syringes	Medical jet(Syria)
8.	Magnetic Hotplate Stirrer	Germany
9.	Burner	Germany

Table (3-4) Tool of Experience

NO.	TOOL	Company\Country
1.	The Flask	China
2.	The Petri Dish	Germany
3.	The Swab	Korea
4.	The Loop	Japan

5.	The Slid	Germany
6.	The Spoon	Germany
7.	The Pasteur /Plastic Pipette	French
8.	Filter Paper	China
9.	The Graduated Cylinder	Germany
10.	The Contain	India
11.	Oral Garage	India
12.	Dissecting Tools	Korea

3.2 Methods

3.2.1: Culture *Lactobacillus*

Step 1:

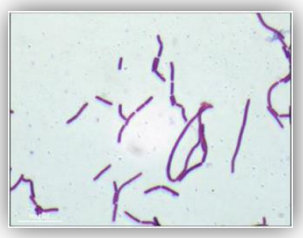
Lactobacillus acidophilus were isolated from yogort, then the bacterial colony was collected and put inside capsules.

Each capsule contain about 2.5 billion CFU

Step 2:-

Under the microscope:

Lactobacillus is a type of bacteria. There are many species of lactobacillus. These are "friendly" bacteria that normally live in our digestive, urinary, and genital systems without causing disease. Lactobacillus is also in some fermented foods like yogurt and in dietary supplements**.+++++**



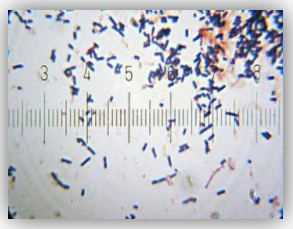


Figure 3(3-1) Lactobacillus Under Microscope

Lactobacilli are a rod-shaped, Gram-positive (crystal violet) , (purple), fermentative, facultative anaerobic . Normally, they form straight rods but under certain conditions spiral forms have been observed. In most cases they form chains of varying length.

Step3:-

Collection growth of *Lactobacillus*:

_After bacterial growth in Nutrient agar, the resulting bacterial colonies are collected using a lube and with the presence of a sterilization hood we collect the colonies and put them in empty capsules and keep them in the refrigerator and thus become ready for dosing,

_The second group of laboratory rabbits is fed (three times) a day after each meal regularly from (probiotic).

3.2.2: Culture *Helicopter pylori*

Step4:

Procedure of *Helicopter pylori*

1-Sample: a stool swab.

2- Blood agar culture medium (which is prepared by 4g of blood agar powder and dissolved in 250ml of D.W and adding some drops of blood).

3-After obtaining the sample and solidification of the culture medium, the sample is transplanted to obtain the growth of colonies of Helicobacter pylori bacteria.

for the procedure of H. pylori culture. P We need the following :-

a_ The bio safety cabinet (hood) :-The UV in the device is turned on fora period of (15 minutes) to sterilize the place of planting before planting to avoid contamination of the culture medium, another microorganism comes.

b_ Loop:- It is used to plan the sample in the dish.

c_ The flame is used inside The (hood) to sterilize the loop.

e_ We take the bean loop from the sample, put the sample in the dish, sterilize the loop and cool it, then draw a layout on the dish using the layout method.

f_ After completing the dish layout, the dish is closed with its lid and turned over.

g_ The culture medium is transferred to incubator and leave it for(24hr).

h_ After the passage of (24hr.) we notice the growth of colonies of pylori bacteria.

*We conduct a chemical test (catalase) to ensure the presence of H.P.

*To perform this test we need the following:-

1-A slide.

2-Sterile loop.

3-Cattlease solution. This test is done inside the test hood by using a loop and taking a small amount of colonies. that has grown and put it in the center of the slide, then using the dropper, and we take a drop of the catalase solution over the H.P sample. We notice that bubbles are an indication of H.P.

Staining the sample:

To perform the staining process, we need the following:-

1-a slide.

2-a gram stain (crystal - iodine - alcohol – safranin)

3-H.P. Sample.

4-loob.

5-drops(NO.4).

Step 5:-

Staining Steps:-

1-Put a small amount of the developing sample on the plate using a loop .

2-Using the first dropper, we fill it with crystal dye and drown the slide, then leave it (2min) and then wash. 2- Using the second dropper, we fill it with iodine dye and drown the slides and then leave it (2min) and then wash.

3-Using the fourth dropper, we fill it with alcohol dye and drown the slides, then leave them (30sec) and then wash.

4- Using the third dropper we fill it with safranin dye and soak the slides and leave(2min) Then wash.

*After staining , the pigments must be fixed on the sample and this is done using the fixation process. This process is done with three passes over the flame.

* After the sample is fixed, it is ready to be viewed under the microscope. It is under the microscope using a 4x lens, then 10x, then 40x, then we put a drop of oil and a 100x lens. HP was seen And clearly.

Step6:-

Under the microscope

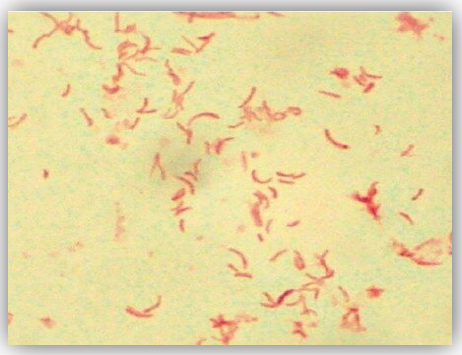


Figure4 (3-2) Helicopter pylori Under Microscope

When viewed under the microscope,

H. pylori may appear to be rod or spiral shaped. Since the bacteria does not retain the primary stain (a characteristic of gram negative bacteria) they will appear reddish/purple in color (color of the counter stain).

Structure of the bacterium

pylori is a spiral-shaped gram-negative bacterium, about 3 micrometers long with a diameter of about 0.5 micrometer. It has 4–6 flagella. It is microaerophilic, i.e. it requires oxygen but at lower levels than those contained in the atmosphere

Does *Helicobacter pylori* have a cell wall

Internal Organization. Thin sections of H. pylori reveal the typical cell wall detail of a gram-negative bacterium that consists of outer and inner, or plasma, membranes separated by the periplasm of approximately 30 nm thickness. The dense cytoplasm contains nucleotide material and ribosomes.

Step 7:-

Dilution of *Helicobacter pylori*

To perform this process:-

we need the following:

1-Palent tube number 9.

2-Distilled water.

3- pipette.

4-hood.

5-flame.

Step 8:-

Work steps:-

1-Fill the planet tube with (9ml) of D.W for each tube and using the flame, the distilled water is heated to sterilize it more precisely.

2-This process is necessary inside the hood. Using the pipette (1ml) of the sample is withdrawn and placed in the first tube, to which distilled water (1ml) is added, and thus the volume is 10ml. We do a good mixing and after good mixing, (1ml) is withdrawn from the first tube and transferred to the tube. The second volume becomes(10ml) also, and after mixing, it is withdrawn (1ml) and transferred to the third tube and we mix and remove 1ml of it and put it in the fourth tube and mix and after mixing it also withdraws (1ml) and transfers the fifth tube and makes a good blending and transfers(1ml)to the sixth tube and mixes well and transfers (1ml)to the seventh tube and mixes (1ml)is transferred to the eighth tube and a good mix is made and transferred to the ninth tube, and the volume becomes (10ml) in the ninth tube. Therefore,(1ml)Is withdrawn and discarded for the purpose of obtaining different concentrations and constant volumes.

* The purpose of dilution: -

Counting the number of colonies that grow in (1ml)of H. pylori bacteria to know the number of Lactobacilli bacteria needed To eliminate the amount of developing colonies 1ml of Helicobacter pylori.

Step9:-

Collection of *Helicobacter pylori*

_After the growth of bacteria on blood agar, the developing colonies of bacteria are collected for the purpose of dosing in two ways (the first method) by lobe and in the presence of a hood and put themin empty capsules and kept in the refrigerator until. the date of dosing is ready for dosing, while (the second way to dosing H. pylori). is to save the HP sample in the broth agar and store it in there frigerator, and after a day or several days, we use a dosing needle and dose the second and third groups of laboratory rabbits and wait for them after several days - a week. Symptoms appeared on the third group and did not appear on the second group that wasdsed with (probiotic).

Among the symptoms that appeared on the third group without aprobiotic._

1-Loss of appetite and inability to eat.

2-General fatigue in the body.

3-An increase in body temperature.

4-Nausea, stomach pain and vomiting.

5-Abdominal bloating.

(While maintaining control of the first group of laboratory rabbits, not giving them neither probiotic nor H.P).

3.3 Dosing and the method of administration of H. pylori bacteria

40laboratory animals were dosed and given to them the H. pylori bacteria (noting that all rabbits were subjected to the same environmental, living and feeding conditions) by an amount of (1 m) per animal for the second and third groups.

3.3.1 Dosing method:-

1-to perform this operation We need (oral cavage) by mouth directly into the stomach.

2-After completing the dosing process, we monitor the health status of all rabbits that were dosed.

3-After a period of time (several weeks) the following symptoms were observed on the third group (without probiotic) without their appearance On the second group with (probiotic).

3.3.2 Killing and dissecting a laboratory animal (The Rabbit).

Method:

The method used to kill the laboratory animal (rabbit) injection method with dilute formalin into the heart

Substance:

Formalin 10% and normal slain 90%

Procedure

We prepare the dose by mixing 10% formalin with 90% normal saline ,and then we hold the laboratory animal (rabbit) and press slightly on the place of the heart. After confirming the injection site, we wait until the heart rate stabilizes, and then we inject the dose into the heart of the laboratory animal (rabbit).

3.3.3 The purpose of killing is to dissect the laboratory animal(rabbit

dissecting a laboratory animal

The laboratory animal was dissected in the usual way of dissection and we removed the digestive system and separated its parts fromthe stomach, small intestine and large intestine, and these parts were examined.

Chapter four

4.1 Distribution of laboratory animal According to Gender.

A total **60** lab. animal were included in our study, the study subjects consisted of **30** male and **30** female with different weight (0.5-2 Kg) as shown in table (4-1).

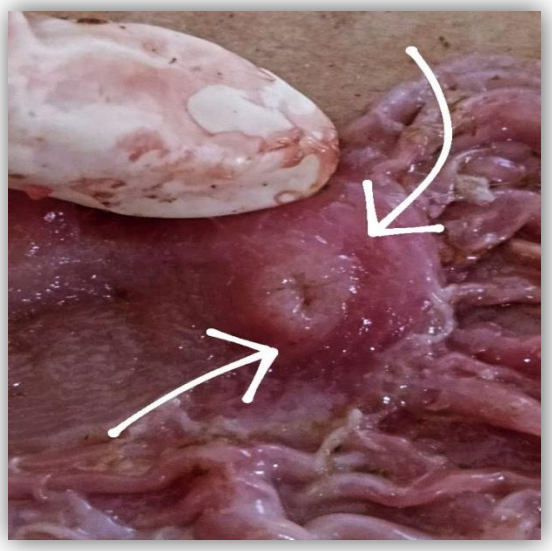
Table (4-1) Distribution of Patients With H.pylori *According* to Gender an weight

Weight groups	Gender		No. of male and female	%
	Male	Female		
0.5-0.9Kg	3	8	11	18.3 %
1-1.5 Kg	22	11	33	55%
1.6-2 Kg	5	11	16	26.7%
Total No.	30	30	60	100 %
Total percentage	50%	50%	100%	

Eleven lab. Animal (18.3 %) in first group with weight (0.5-0.9 Kg), while 33 lab. Animal (55%) were present with weight (1-1.5 Kg), and 16 lab. animal (26.7%) were included as shows in this table (4-1).

Table (4-2) differentiate between all three groups of lab animals use in this study

Symptoms	**Group 1**	**Group 2**	**Group 3**
	Lab. Animal with H. pylori	**Lab. Animal with H.pylori + probiotic**	**control**
Abdominal bloating Nausea and vomiting Weight loss for no reason Not feeling hungry Feeling tired for no reason Fever	Present	present It is mild and) disappears after a (short period of time	***Normal***
Symptoms that appeared in the mouth Change the color of the gums and tongue from the natural color to blue-white or bluish-white .	Present	Absent	Absent
pharynx	***Normal***	***Normal***	***Normal***
Esophagus	***Normal***	***Normal***	***Normal***
Inflammation and ulceration of the stomach lining	Present	Absent	Absent
Inflammation and necrosis of the small intestine	Present	Absent	Absent
Inflammation and necrosis of the large intestine	Present	Absent	Absent



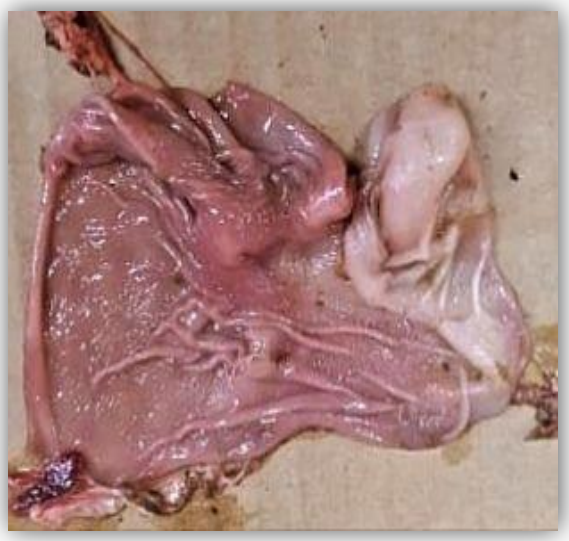


Figure6(4-1) **Figure6(4-2)**

After autopsy of the animals that were given H. Pylori only and the animals that were given H. Pylori and a probiotic together, it was found that the animals that were not given the probiotic developed peptic ulcer. H. pylori bacteria usually live in the mucous layer that covers and protects the tissues lining the stomach and small intestines.

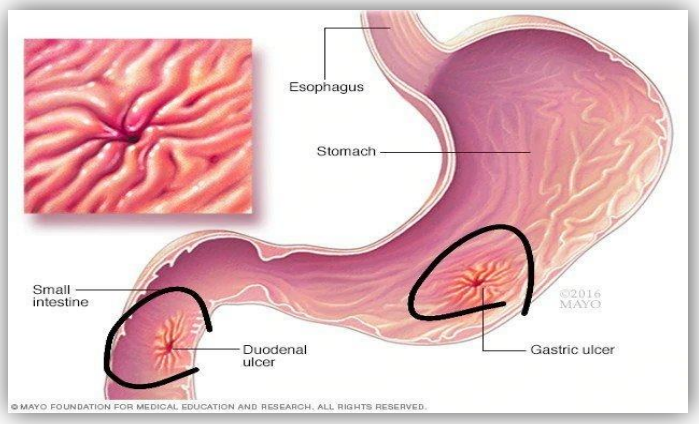


Figure7(4-3)

So peptic ulcer :- is a wound that appears, usually, in the inner membrane of the stomach wall or in the upper part of the small intestine (which is the part called the duodenum). Peptic ulcers form when the protective membranes of the stomach or intestines are damaged. The animals that took the probiotic were healthy and there was no stomach damage. As shown in the figures above.

4.2 Result and Discussion

Current antibiotic regimens against *H pylori* infection may be effective, but complex dosing and development of resistance are always concerns. Animal studies and limited clinical trials of *H pylori* antigens have been conducted, with no final conclusive findings. A number of data now exist, supporting the potential role of probiotic for protection of GIT against *H pylori*.

However, we are still at a preliminary stage in clinical development. The best immunogens, the best mode of presentation, the number of doses needed, optimal age at immunization, expected benefit, cost-effectiveness, and other factors involved in vaccine development require further study(**Monath et al., 1998**). The present study employed rabbits to assessed the effects of live probiotics on *H. pylori* growth, adherence to gastric epithelial cells and *H. pylori*-induced inflammation.

The findings demonstrated that all lab animal in group 1 shows a significant effect (100%) with *H. pylori* dose and the lesion are appeared in gastro intestinal tract especially in stomach as compared with control group.

While in Group 2 there is no significant change in digestive system as compared with control group.

This result may be due to that *L. acidophilus* significantly inhibited the adherence of *H. pylori* to GES-1 cells and also decreased IL-8 production by GES-1 cells following stimulation with HP-LPS. In addition, *L. bulgaricus* inhibited the TLR4/IκBα/NFκB signaling pathway in a time-dependent manner (**Song *et al*., 2019**).So the probiotic act as umbrella and protect the digestive system from various pathogen including H. pylori. It is well established that adhesion to mucosal surfaces is a key step in the pathogenesis of *H. pylori*.

The inhibition of *H. pylori* colonization by probiotics is strain specific. Chen *et al*reported that, live and dead *lactobacilli* inhibits *H. pylori* adhesion to SGC7901 cells. Hsieh *et al* identified that *L. johnsonii* MH-68 and salicinius AP-32 effectively suppress *H. pylori* viability and reduce *H. pylori* colonization in the gastric mucosa of mice. Aiba*et al* proved that *L. salivarius* is capable of producing a high amount of lactic acid and inhibiting the growth of *H. pylori*.

The inhibitory effect of probiotic pre-treatment against *H. pylori* adherence is likely mediated via increasing production of mucin (Mack *et al*., 1999), or competition to bind *H. pylori* adhesion sites by probiotics (Valeur*et al*., 2004).

The adhesion of *H. pylori* to GES-1 cells was not inhibited when cells were treated with probiotics post-infection. Therefore, it appears that probiotics cannot reduce the adhesion rate of *H. pylori* when it is already adhered to gastric epithelial cells. Therefore, probiotics may be more effective in a preventive rather than therapeutic role.

Certain probiotic strains produce bactericidal substances that are either secreted into the culture supernatant or expressed on the cell surface, which can significantly inhibit *H. pylori* (El-Adawi*et al*., 2013). Therefore a possible mechanism of action is the secretion of lactic acid by the probiotics, as the metabolic end products of lactic acid fermentation and organic acids are capable of interfering with the growth of pathogens. Notably, El-Adawi*et al* demonstrated that lactic acid may be a potent antimicrobial.

Rabbit immunity plays an important role in the development of clinical diseases. Pro-inflammatory cytokine production occurs during *H. pylori* infection, with the inflammatory reactions potentially leading to chronic inflammation rather than eliminating *H. pylori* (Jang *et al*., 2017).

The transcription factor NF-κB can be activated by IL-1β, LPS, peptidoglycan and tumor necrosis factor-α during *H. pylori* infection (Lee *et al*.,2005).NF-κB is critical modulator of cytokine expression (Cha *et al*., 2015). TLRs are cell transmembrane and pathogen-associated molecular pattern receptors that have a central role in the recognition of microbial pathogens and may be a first line of immunity against *H. pylori* (Villena*et al*., 2013). HP-LPS-induced inflammation in gastric mucosa demonstrates similar pathological characteristics to the mucosal inflammation initiated by *H. pylori* infection (Stein *et al*., 2017).

The present study suggested that suppression of the TLR4/NF-κB signaling pathway occurred in a time-dependent manner and was mediated through the stabilization of IκBα.

In conclusion, the present study identified that probiotic strains were effective in reducing the *H. pylori* load. Considering the safety and health function of probiotics, food containing *L. acidophilus* and *L. bulgaricus* may have potential as an adjuvant therapy for gastric diseases caused by *H. pylori*, and displays promise as a preventive measure against *H. pylori* infection.

Chapter five

5.1 Abstract

Background:-pylori (Latin: Helicobacter pylori) or gastrobacterium is a type of bacteria of small, air-friendly Gram-negative bacilli that colonize the gastric and duodenal mucosa, causing mucositis, and are associated with the development of peptic ulcers in the stomach and duodenum and gastric cancer. More than 80% of people infected with MRSA remain without symptoms or complications. About 50% of the world's population carries this bacteria, making it the most common infection in the world and its prevalence is greater in developing countries than in developed countries. Countries. The route of infection is completely unknown .

The animals used in the experiment(Expermintal animal model)

Laboratory animals were used, which were divided into two groups. A control group consisting of 20 animals was used as comparison animals at the autopsy of each group. The treated group of 40 animals was divided into two groups. The first group consisted of 20 animals tested with the bacterial vaccine, and the second group consisted of 20 were tested with the bacterial vaccine in addition to the probiotic .

5.2 Research Recommendations : -

1 - We recommend researchers, scientists and the bodies responsible for producing medicines to pay attention to lactobaclus bacteria and use them as a treatment to get rid of H.P diseases .

2 - One of the benefits of this treatment is that it is cheap and available.

3 -Therefore, we advise ordinary people to eat a lot of yoghurt because it contains a high percentage of Lactobacilus

4 - Despite the severity of H.P pathology, the risk of infection and the severity of the pain experienced by the H.P patient, we can reduce this disease by 99%, through the production of a (probiotic) treatment, which is (Lactobacili).

5 - This treatment is produced by taking a sample of yoghurt and planting it in a suitable medium (Nutrient agar), collecting the resulting colonies and filling them in capsules, which are taken three times before each meal

6 – We as Laboratory, advise ordinary people who are not related to this field or the elderly not to take any antibiotics for their negative effects on our lives and for our body's resistance to some diseases, as these antibiotics can cause a weakening of the immune system and our body may reach a stage that resists all antibiotics There is no treatment left to get rid of a specific disease, so the fate will inevitably be death

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