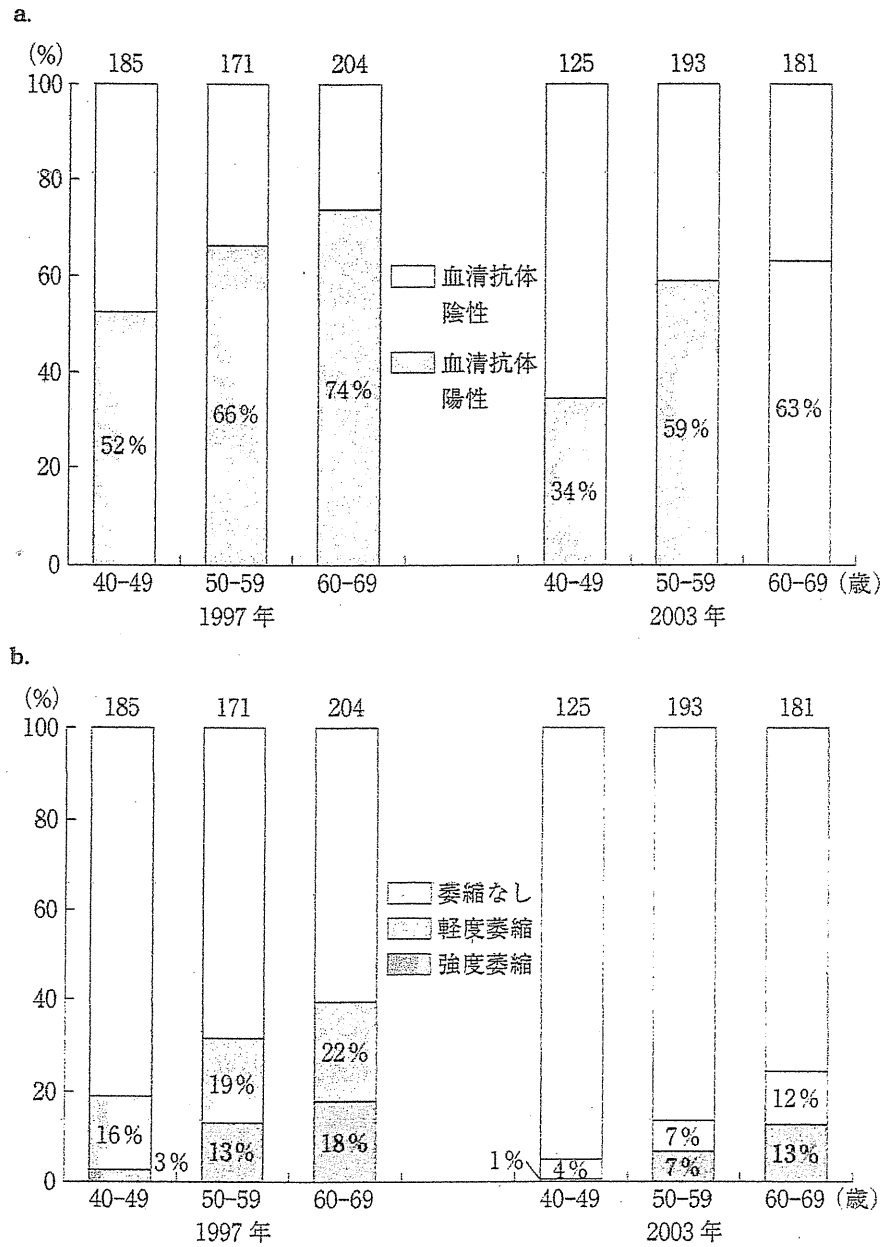

図3　年齢別の*H. pylori*抗体陽性(感染)率(a)と血清学的胃粘膜萎縮の頻度(b)

北海道A町の基本健診(当時)の受診者の血清を測定した．感染率はJ-HM-CAP(国内由来の菌株による．現在は製造中止)で測定し，添付文書記載の2.3以上を陽性とした．上段の数字は対象者数．血清学的胃粘膜萎縮の判定は，PG I≦50 ng/mLかつ，PG I/PG II≦2.5のとき強度萎縮，それ以外でPG I≦70 ng/mLかつ，PG I/PG II≦3.0のとき軽度萎縮．

ので，未感染者では胃粘膜萎縮はまれである．胃粘膜萎縮の頻度は*H. pylori*感染率の低下に伴って，低下することが予想される．

図3に同一地域の健診受診者の血清抗体陽性(*H. pylori*感染)率と血清学的胃粘膜萎縮の頻度を示す．同じ年齢で比較すると6年間で*H. pylori*感染率，胃粘膜萎縮の頻度とも低下している．*H. pylori*感染率の低下に比べて，胃粘膜の萎縮の頻度の低下は，より急速である．胃粘膜萎縮の頻度低下の主な原因は*H. pylori*感染率の低下であるが，食生活の変化などの影響も考えられる．1960年以降に生まれた世代では，それ以前に生まれた世代に比べ，高塩食品の摂取は少ないと考えられる．我が国では，以前に比べて炭

水化物の摂取が減少して，脂肪分の摂取が増加している．炭水化物，特に米飯を多く摂取する食生活では高塩食品が好まれるが，欧米化した脂肪分の摂取が多い食生活では高塩食品の摂取は減少する．

また，推論であるが感染時期が遅れていることも原因として考えられる．上水を介しての感染では，1歳に達する前後には，ほとんどの感染が起こっていると考えられる．一方，家族間感染の場合には5歳に達するまでの遅い時期に成立する持続感染が少なくないと考えられる．この感染時期の違いは，成長過程でもあるので，胃粘膜萎縮に与える影響が意外に大きい可能性がある．

今後も，*H. pylori* 感染率の低下は続くと考えられる．胃粘膜の萎縮を促進する高塩食品の摂取や喫煙が増加することは考えにくいので，我が国の胃粘膜萎縮の頻度は，若い世代ほど低くなっていくと予想される．言い換えると，若い世代ほど胃粘膜の健康度は良くなるということである．**図3**に示すように*H. pylori* 感染率に比べて，胃粘膜萎縮の頻度は低くなっている．我が国の胃粘膜萎縮の頻度は年齢ごとに*H. pylori* 感染率よりも多少低い値で，若い年代の方が感染率との乖離が大きいと推測される．

H. pylori 感染率，胃粘膜萎縮頻度の低下によって，既に減少傾向を示している胃癌の罹患，死亡は今後も減少を続けると考えられる．*H. pylori* 未感染者では，胃粘膜の萎縮が起こることはまれである．対策が必要となるのは*H. pylori* 感染者の成人である．除菌治療後にも胃癌は発生し[14]，治療時に胃粘膜萎縮が強いほど，発生が高頻度であることが明らかになっており[15]，今後の課題である．

おわりに

我が国では，*H. pylori* 感染率の低下により胃粘膜萎縮の頻度も低下している．その結果，胃癌は減少しつつあるが，*H. pylori* 感染があって胃粘膜の萎縮が進んだ成人の胃癌対策が，なお課題となっている．

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II 胃癌の疫学

胃癌の危険因子

H. pylori 感染

H. pylori infection

菊地正悟

Key words：mortality, incidence, promoter, natural history

はじめに

Helicobacter pylori（以下ピロリ菌）は胃癌の原因の一つであることが明らかにされた．これまでの研究結果から，ピロリ菌が発がんに与える影響について，胃癌の自然史（経過）の各段階で，どのような影響を与えているのか，主に疫学研究を振り返りながら検討する．

1 胃癌罹患への影響

ピロリ菌と胃癌の関係は，表1に示す研究[1-8]などで明らかにされてきた．これらの研究は，ピロリ菌感染の有無がその後の診断可能な臨床的な胃癌発生に，どのように影響したかを分析したものである．

1）胃　癌

当初，相対危険度は感染者と一度も感染したことのない未感染者で6倍程度と考えられていた[1,2]．しかし，自然除菌による過小評価などの影響が明らかになっている[9]．内視鏡検査を含めて，未感染を確認した対照と感染者を比較した研究[3]や，自然除菌後に長く残存するピロリ菌の毒素CagAに対する抗体をウェスタンブロットで測定した研究[8]で，感染者と未感染者のリスクの違いは少なくとも20倍以上であるという結果が得られている．

2）分化型胃癌と未分化型胃癌

胃癌は，分化型胃癌と未分化型胃癌に大別される．当初は，慢性胃炎によって萎縮した粘膜から主に発生する分化型胃癌との関連が強いと考えられたが，ピロリ菌とそれぞれのタイプの胃癌との関係の強さに差がないことが明らかになっている[10]．

除菌治療が保険適用となり，広く行われるようになると，除菌後に発生する胃癌が問題となる．除菌後も未感染者に比べて胃癌のリスクは高いので，経過観察が必要である．臨床がんの発生を促進する程度は同じでも，除菌時以後の臨床がんに至る過程が分化型がんと未分化型がんで異なる可能性もある．除菌後にどのようなタイミングで，どのような胃癌が発生するかについては，今後のデータの集積が必要である．

3）近位部（proximal）胃癌と遠位部（distal）胃癌

欧米では，近位部（噴門部）の胃癌はピロリ菌感染と負の関連を示すことが報告されている[11]．我が国では同様の報告はないが，近位部胃癌は遠位部胃癌に比べて感染者と非感染者のリスク比が小さい[12]．これらの事実は次のように説明できる．近位部にはピロリ菌感染者でリスクが高いタイプと，非感染者でリスクが高いタイプ

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表1 ピロリ菌と胃癌の関係を分析した主な疫学研究

著者	地域	年齢(平均)	追跡期間(年)	オッズ比(95%信頼区間)	陽性率 罹患例	陽性率 対照	文献
前向き研究							
Nomura et al.	ハワイ	46-65(59)	13.0	6.0(2.1-17.3)	94%	76%	1)
Parsonnet et al.	カリフォルニア	(54)	14.2	3.6(1.8-7.3)	84%	61%	2)
Uemura et al.	広島		7.8	∞(1.7-∞)	100%	81%	3)*
Sasazuki et al.	日本	(57)	11.0	5.1(3.2-8.0)	94%	75%	4)
症例対照研究							
Asaka et al.	北海道	(60)		2.6(1.5-4.3)	88%	75%	5)
Kikuchi et al.	東京	20-39(34)		13.3(5.3-35.6)	89%	39%	6)
Kikuchi et al.	東京	20-29		7.0(0.7-69.2)	100%	28%	7)
		30-39		14.5(4.3-49.2)	90%	43%	
		40-49		9.1(4.8-17.1)	95%	54%	
		50-59		3.5(2.1-5.9)	88%	71%	
		60-69		1.5(0.9-2.5)	87%	81%	
Ekstrom et al.	スウェーデン	(67)		21.0(8.3-53.4)	93%	59%	8)
		40-59		22.1(4.5-108.2)	88%	32%	
		60-69		19.2(4.3-86.3)	91%	57%	
		70-79		24.6(5.5-109.2)	95%	68%	

下記以外は血清IgG抗体

*IgG抗体，病理組織学検査，迅速ウレアーゼ検査

の2種類の胃癌が発生する．我が国ではピロリ菌感染率が高いために，近位部の胃癌もピロリ菌感染者でリスクが高いタイプが大部分となる．しかし，まれに非感染者にリスクが高いタイプも発生しているため，全体でみると，遠位部に比べてリスク比が小さくなる．

2 ピロリ菌のプロモーター(promoter)作用

スナネズミを用いた実験で，ピロリ菌は胃癌発生のプロモーターとして作用することが明らかになっている[12]．動物実験の結果であるという問題はある．しかし，発がんへの作用でヒトとスナネズミで大きな違いがあるとは考えにくいことから，ピロリ菌はプロモーターとして作用すると考えられる．ニトロソアミンなどのinitiator(イニシエーター)への曝露が日常的な胃では，強力なプロモーターであるピロリ菌の存在だけで，胃癌の発生が著増することは十分考えられる．

早期胃癌内視鏡切除後に，ピロリ菌の除菌を行うと異所再発が抑制されるという結果を報告した[13]．この研究は，内視鏡で診断できない小さな潜在がんが，診断可能な臨床がんに育つ過程で，ピロリ菌が影響することを示したものである．ヒトでのプロモーター作用を示唆する研究と考えられる．

3 自然経過の各段階でのピロリ菌の影響

早期胃癌内視鏡切除後の研究では，ピロリ菌を除菌しても同所再発は抑制されなかった．同所再発は，臨床がんまで成長した胃癌の一部が残されて，再び大きくなったものと考えられる．異所再発の抑制と合わせて考えると，潜在がんが臨床がんに至る過程はピロリ菌の存在で成長が促進されるのに対して，臨床がんまで成長するとピロリ菌の影響を受けずに成長するようになると推論される．この段階での除菌による抑制効果は1/3くらいである．

一方，臨床がんに成長するまでには，ピロリ菌感染者と未感染者で20倍以上のリスク比が

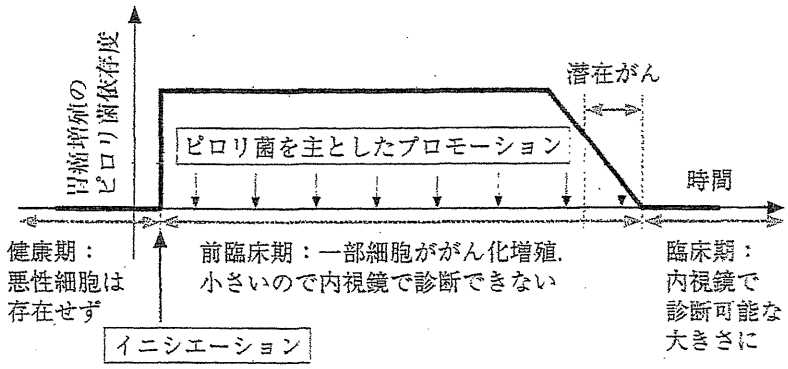

図1 胃癌の自然史（自然経過）とピロリ菌の作用
詳細は本文を参照．

ある．潜在がんになるまでに20÷3=6.7倍以上の影響を与えていることになる．

ピロリ菌がプロモーターとして，胃癌の成長にどのように影響するかを模式的に示したのが**図1**である．イニシエーションまでは作用を及ぼさず，その後プロモーター作用を発揮して胃癌の成長を促進し，潜在がんから臨床がんになる頃に影響はなくなると考えられる．臨床がんになってからは自律増殖するので，ピロリ菌の影響はないと考えられる．イニシエーションから作用が終わるまでの影響の強さについてはデータが全くないので，ある時点まで一定とし，その後漸減して0になるように図示した．作用が終わる過程での漸減は，ある時点で突然影響が0になることは考えにくいことと，個々の胃癌によって影響が0になる時点が多少異なる可能性を考えたためである．

4 ピロリ菌感染率の減少と胃癌の減少

我が国では，後から生まれた世代ほどピロリ菌感染率が低下している．ピロリ菌は，大部分の感染が5歳までに起こるとされているので，小児期の衛生状態の改善がピロリ菌感染率の低下をもたらしている．2010年の感染（有病）率の推定値は，40歳代で32％，50歳代で43％，60歳代で56％，70歳代で70％となっている[14]．この結果として，厚生労働省の人口動態統計のデータから計算した10歳階級ごとの胃癌死亡率は年々低下している（**図2**）．死亡率は治療の影響も受けるが，強力な発がん促進要因であるピロリ菌感染率の低下が罹患率を低下させていることが，死亡率の低下の主な原因であると考えられる．

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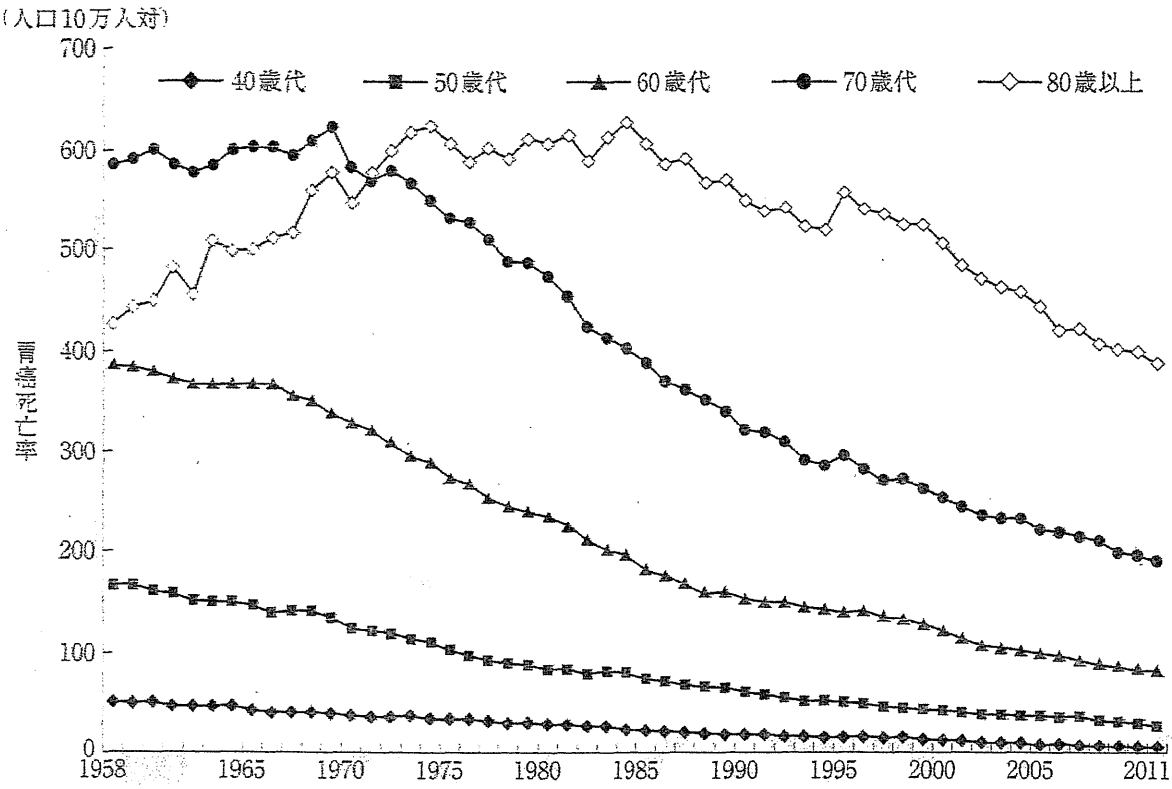

図2 我が国の胃癌死亡率(男性)の推移

40～80歳以上まで、10歳階級ごとに男性の死亡率を示した。60歳以下では、継続して低下している。一方、70歳代では1970年頃から低下し、80歳以上では1975年頃まで上昇し、1985年頃から低下している。

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ピロリ菌除菌の費用対効果

——除菌によって削減できる胃がん，消化性潰瘍の医療費

Cost and effectiveness of *Helicobacter pylori* eradication

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◎2010年のわが国人口の20～69歳の各年齢層の20%(5人に1人)を対象にピロリ菌の感染検査を行い，陽性者の除菌をするというモデルによって，要する費用と削減できる医療費を検討した．除菌に要する費用は約910億円で，胃がんでは889億円，消化性潰瘍で29億円の医療費削減効果が期待でき，差し引き8億円程度の黒字が予想された．これ以外のプラス面として血小板減少性紫斑病，胃のMALTリンパ腫，鉄欠乏性貧血の減少がある．マイナス面として除菌の副作用のほか，未観察であるが逆流性食道炎や食道下部腺がん，胃噴門部がんの発生の増加が考えられる．胃がんの救命，消化器症状などによる病悩期間の減少，次世代の感染減少もプラス面と考えられるので，全体としてはピロリ菌の除菌は費用に比べて効果が大きいと考えられる．

Key word：経済評価，ピロリ菌除菌，胃がん，消化性潰瘍，除菌副作用

Helicobacter pylori(以下，ピロリ菌)の除菌によって，消化性潰瘍だけでなく胃がんの発生も減少することが明らかになった．胃がんの予防として除菌を考える場合に，除菌に要する費用と胃がん予防効果の比較は重要である．2013年2月からピロリ菌感染胃炎の除菌治療が医療保険の対象となったことにより，ほとんどのピロリ菌感染症が，内視鏡検査で胃炎を確認すれば保険で治療できるようになった．今後は除菌治療を受けた人の増加によってデータが集積されることが期待される．

しかし，これまでは消化性潰瘍など一部の疾患を除くとデータの集積はかならずしも十分でない．除菌後の胃がん発生率，胃がんの進行速度，対象者の受療行動について，ある程度のことは明らかになりつつあるが，いまだデータが不十分である．このような制約はあるが，使用可能なデータにもっとも確実あるいは真実に近いと考えられる仮定のもとに，ピロリ菌の除菌に要する費用と期待される胃がんと消化性潰瘍の予防による医療費削減効果を試算した．

効果の評価の指標

わが国におけるピロリ菌除菌による胃がん予防効果に関する分析はこれまで，費用対医療費削減効果として実施されたものがほとんどである[1,2]．Takeらの報告[3]や，早期胃がん内視鏡切除後であるが無作為割付による研究[4]から，除菌によって胃がんの発生は約1/3になることが報告されている．このように，除菌後にも少なからず胃がんは発生する．また，定量的な十分なデータではないが，除菌に成功すると定期検査に訪れる頻度が減少することが明らかになっている．

表 1　費用と胃がん予防効果の計算に用いた前提

①	これまでの研究から推定した2010年の *H. pylori* 陽性率(表2)を，70歳未満の陽性率とした
②	5歳階級ごと，2011年の人口動態の死亡率(表3)で人口が減少していくものとして，5年ごとに計算した
③	*H. pylori* 感染者の胃がん罹患率には，1975年の地域がん登録データによる推計の胃がん罹患率データ(表3)を使用した
④	*H. pylori* 感染検査は管理費を含めて1,000円/人，二次除菌も考えて除菌費用は14,000円/人とした
⑤	1人当り胃がん治療費を1,425,532円とした
⑥	胃がん予防効果は表2の数値を用いた

表 2　分析に用いたピロリ菌陽性率と予防率*

年齢(歳)	ピロリ菌陽性率(%)	予防率(%)	検査対象者数(人)
20～24	8.6	91	1,285,200
25～29	11.1	87	1,458,800
30～34	16.0	83	1,668,200
35～39	21.0	79	1,957,200
40～44	25.9	75	1,748,200
45～49	32.1	70	1,606,600
50～54	39.4	66	1,529,000
55～59	45.9	62	1,732,600
60～64	51.5	58	2,007,400
65～69	58.6	54	1,642,000
合計	—	—	16,635,200

*：除菌によって胃がん発生が予防される割合．

胃がんの予防効果と医療費削減効果

1. 計算方法

20～69歳の5歳階級ごとに表1に示す前提によって，2010年の日本の人口の20%(表2)を対象に除菌を行った場合の費用と期待医療費削減効果を計算し，各階級分を合計した．死亡率[5](表3)で人口の減少を計算するのは，胃がん以外の疾患や事故などでも死亡する人があるので，年を経るにつれて人口が減少することを考慮するためである．用いたのは生命表の考え方であるが，生命表では1年ごとに人口を計算するのに対し，ここでは5年ごとの計算とした．ピロリ菌感染者の胃がん罹患率に1975年の罹患率[6](表3)を用いた理由は，この時代は人口のほとんどがピロリに菌に感染したことのある“飽和状態”であったと考えられるためである．除菌費用を14,000円/人としたが，内視鏡検査の費用は含めていない．

1人当り胃がん治療費を1,425,532円としたのは，浅香の報告[1]から1人当りの胃がん治療費を計算した．除菌による胃がん予防効果は20歳未満を95%(除菌で胃がんは1/20になる)，70歳以上を50%(同1/2になる)として，その間が直線的になるようにした．

以上のデータを用い，除菌後90歳になるまでの累積の胃がん罹患数を計算し，予防率とのかけ算で，期待胃がん減少数を計算した．これに1人当り治療費をかけて期待医療費削減効果とした．20～69歳の結果を合計して総費用と総効果を計算した(「サイドメモ」参照)．

2. 結果

検査と除菌に要する費用と，陽性者の除菌に要する費用の合計(総費用)は約910億円で，期待される医療費削減効果(総効果)は約889億円であった．

3. 検討

計算では総費用が総効果を上まわった．しかし，救命による経済損失回避は計算に入れていない．このため，実際の効果はより大きくなるはずである．また，ピロリ菌の感染経路は家族内感染，とくに母子感染が主であることが明らかになって

表 3　計算に用いた死亡率，胃がん罹患率

項目	死亡率		胃がん罹患率	
暦年	2011		1975	
性別	男	女	男	女
20～24 歳	62.9	32.5	2.8	3.0
25～29 歳	67.0	37.6	5.4	8.1
30～34 歳	78.2	45.9	12.6	16.6
35～39 歳	102.5	63.7	25.7	24.4
40～44 歳	153.1	90.5	48.9	38.9
45～49 歳	240.0	141.5	97.5	47.9
50～54 歳	391.5	203.0	151.1	73.0
55～59 歳	615.4	295.1	231.5	98.3
60～64 歳	959.7	414.2	356.4	161.6
65～69 歳	1,512.9	624.4	512.3	206.5
70～74 歳	2,263.3	990.8	673.4	272.3
75～79 歳	3,939.4	1,810.7	735.3	335.9
80～84 歳	7,067.3	3,520.4	762.3	328.8
85～89 歳	11,955.9	6,928.0	565.0	303.9

注：いずれも人口 10 万人対．

いる．除菌により次世代のピロリ菌感染も減少すると考えられることから，効果はより大きくなると考えられる．

消化性潰瘍の予防効果と医療費削減効果

1．計算方法

消化性潰瘍については利用できるデータが胃がんよりも限られる．非常におおまかな予防効果の推定となるが，以下のような方法で予防による医療費削減効果を推定した．あくまでの参考値と考えていただきたい．

罹患数調査がないので，患者調査[7]による通院数を有病数として有病率を計算した．有病率はピロリ菌陽性率に比例するので，除菌が保険適用となる以前の 1996 年の有病率（表 4）を 1996 年と 2010 年のピロリ菌陽性率で補正して算出した．部位（胃，十二指腸，不明）は考慮しなかった．

2010 年の人口の 20％を検査と除菌した（胃がん対策で実施したので追加費用は 0）として，この対象の 5 歳階級ごとの 20～69 歳の有病数から除菌しなかった場合の 90 歳までの医療費を推定した．対象はがんの分析と同様に 2011 年の死亡率[5]で死亡し，1 年後には 90％が再発（10％が治癒）して 1 年間 1 人平均 10,000 円の薬剤治療[8]と年 1 回内視鏡（約 11,000 円）受けることとした．

2．結果と検討

医療費の節約効果は 29.4 億円であった．ここでの計算では治療が必要な時期の内視鏡検査を 1 年 1 回としたが，実際にはそれより多いと考えられるので，節約効果はより大きくなると考えられる．再発率（翌年治療が必要となる率）を 90％としたが，これに関しては信頼できるデータをみつけることができなかった．胃潰瘍と十二指腸潰瘍で異なることも考えられる．再発率を 80％とした計算も行ったが，その場合の医療費の節約効果は 19.3 億円となった．

ピロリ菌除菌とその他の疾患の関係

ピロリ菌が引き起こす疾患として，胃がんと消化性潰瘍に以外に，保険適用となっている血小板減少性紫斑病[9]と MALT リンパ腫[10]がある．鉄欠乏性貧血についても，ピロリ菌を除菌すると一部で貧血の改善がみられる[11]ことが明らかになっている．これらの疾患は数は少ないが，ピロリ菌を除菌することで，発生が予防されて医療費が削減できる．

一方，逆流性食道炎の一部，食道下部腺がんや

サイドメモ　割引率（discount rate）

除菌に要する費用（現在の支出）と将来節約できる医療費（除菌しない場合に必要となる医療費＝将来の支出）を比較する場合に，通常の経済分析では割引率を考慮して計算する．現在支出しなければ，支出しなかった金額に利子がつくので，将来の支出はその分だけ割安になることを考慮する必要があるためである．これまで，わが国はデフレーションの継続により金利が 0 に近い状態が続いていたが，日本銀行が行っている債権などの購入による円の大量供給（いわゆる異次元緩和）により，今後は金利が上昇する過渡期にある．本稿のモデルでは数十年先の医療費の節約を考えている．それまでの期間に割引率の値をどう決めるかについて，現時点では予測がきわめて難しい．割引を 3％程度とし，1～7％それぞれの場合についても計算するのが一般的であるが，煩雑になるのを避けることもあり，ここでは割引率を考慮せずに計算した．

表 4　1996年の消化性潰瘍の有病率

病名	胃潰瘍		十二指腸潰瘍		部位不明の消化性潰瘍	
性別	男	女	男	女	男	女
20～24歳	14.0	16.7	6.0	4.2	2.0	0.0
25～34歳	30.8	22.2	19.4	4.7	2.3	1.2
35～44歳	89.8	43.1	41.2	12.3	7.3	1.2
45～54歳	144.5	68.0	42.7	17.3	7.1	4.1
55～64歳	208.6	99.2	41.7	21.1	10.4	2.5
65～69歳	259.3	149.9	45.4	23.1	9.7	5.8
70～74歳	306.7	180.9	42.5	17.4	14.2	3.5
75～79歳	319.8	228.5	31.2	23.8	7.8	4.8
80～84歳	258.5	254.9	23.5	19.6	11.8	6.5
85歳以上	235.3	209.4	39.2	16.8	0.0	8.4

人口10万人対.

胃噴門部がんについては，ピロリ菌感染が発生を予防すると考えられている[12]．これらの疾患のうち，がんについて地域がん登録によるモニタリングが行われているが，わが国ではこれまでのところ発生の増加は観察されていない．万一，これらの疾患がピロリ菌の除菌によって増加するようであれば，除菌のマイナス面として考慮する必要がある．

また，まれであるが，除菌の治療ではペニシリンアレルギーなどにより入院が必要となる程度の副作用が報告されている．このような例も除菌のマイナス面として考慮する必要がある．

全体としての除菌の経済評価

2010年の日本の20～69歳の人口の各年齢層の20%を対象にピロリ菌の感染検査を行い，陽性者を除菌するというモデルを考えた．除菌に要する費用は約910億円で，胃がんでは889億円，消化性潰瘍で29億円の医療費削減効果が期待でき，差し引き8億円程度の黒字が予想できるという結果であった．前項で述べた各疾患の影響は考慮しない結果であるが，胃がんの救命による効果や，消化器症状などによる病悩期間が減少する影響，次世代の感染減少も考えると，ピロリ菌の除菌は費用に比べて効果が大きいと考えられる．

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Helicobacter. 2014 Mar 28. doi: 10.1111/hel.12127. [Epub ahead of print]

Development of New PCR Primers by Comparative Genomics for the Detection of Helicobacter suis in Gastric Biopsy Specimens.

Matsui H[1], Takahashi T, Murayama SY, Uchiyama I, Yamaguchi K, Shigenobu S, Matsumoto T, Kawakubo M, Horiuchi K, Ota H, Osaki T, Kamiya S, Smet A, Flahou B, Ducatelle R, Haesebrouck F, Takahashi S, Nakamura S, Nakamura M.

Author information

Abstract

BACKGROUND: Although the infection rate of **Helicobacter suis** is significantly lower than that of **Helicobacter** pylori, the H. **suis** infection is associated with a high rate of **gastric** mucosa-associated lymphoid tissue (MALT) lymphoma. In addition, in vitro cultivation of H. **suis** remains difficult, and some H. **suis**-infected patients show negative results on the urea breath test (UBT).

MATERIALS AND METHODS: Female C57BL/6J mice were orally inoculated with mouse **gastric** mucosal homogenates containing H. **suis** strains TKY or SNTW101 isolated from a cynomolgus monkey or a patient suffering from nodular gastritis, respectively. The high-purity chromosomal DNA samples of H. **suis** strains TKY and SNTW101 were prepared from the infected mouse **gastric** mucosa. The SOLiD sequencing of two H. **suis** genomes enabled comparative genomics of 20 **Helicobacter** and 11 Campylobacter strains for the identification of the H. **suis**-specific nucleotide sequences.

RESULTS: Oral inoculation with mouse **gastric** mucosal homogenates containing H. **suis** strains TKY and SNTW101 induced **gastric** MALT lymphoma and the formation of **gastric** lymphoid follicles, respectively, in C57BL/6J mice. Two conserved nucleotide sequences among six H. **suis** strains were identified and were used to design diagnostic **PCR** primers for the **detection** of H. **suis**.

CONCLUSIONS: There was a strong association between the H. **suis** infection and **gastric** diseases in the C57BL/6 mouse model. **PCR** diagnosis using an H. **suis**-specific primer pair is a valuable **method** for detecting H. **suis** in **gastric biopsy specimens.**

KEYWORDS: Helicobacter suis , **PCR** diagnosis, comparative genomics, **gastric biopsy** specimen, mouse infection model, next-generation sequencing

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Analysis of the microbial ecology between *Helicobacter pylori* and the gastric microbiota of Mongolian gerbils

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Animal models are essential for *in vivo* analysis of *Helicobacter*-related diseases. Mongolian gerbils are used frequently to study *Helicobacter pylori*-induced gastritis and its consequences. The presence of some gastric microbiota with a suppressive effect on *H. pylori* suggests inhibitory gastric bacteria against *H. pylori* infection. The aim of the present study was to analyse the microbial ecology between *H. pylori* and the gastric microbiota of Mongolian gerbils. Gastric mucosa samples of *H. pylori*-negative and -positive gerbils were orally inoculated to five (Group 1) and six (Group 2) gerbils, respectively, and the gerbils were challenged with *H. pylori* infection. The colonization rate (40 %) of *H. pylori* in Group 1 gerbils was lower than the rate (67 %) in Group 2 gerbils. Culture filtrate of the gastric mucosa samples of Group 1 gerbils inhibited the *in vitro* growth of *H. pylori*. Three lactobacilli species, *Lactobacillus reuteri*, *Lactobacillus johnsonii* and *Lactobacillus murinus*, were isolated by anaerobic culture from the gerbils in Groups 1 and 2, and identified by genomic sequencing. It was demonstrated that the three different strains of lactobacilli exhibited an inhibitory effect on the *in vitro* growth of *H. pylori*. The results suggested that lactobacilli are the dominant gastric microbiota of Mongolian gerbils and the three lactobacilli isolated from the gastric mucosa samples with an inhibitory effect on *H. pylori* might have an anti-infective effect against *H. pylori*.

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INTRODUCTION

Helicobacter pylori is one of several bacterial microbiota capable of colonizing the human stomach (Bik *et al.*, 2006). Whilst most individuals remain asymptomatic (Aviles-Jimenez *et al.*, 2004), ~15 % of *H. pylori* infections result in peptic ulcers and 0.5–2 % of infected individuals develop gastric adenocarcinoma (Atherton, 2006). It has been estimated that *H. pylori* colonization increases the risk of gastric cancer ~10-fold (Suzuki *et al.*, 2007).

There have been many challenges to establishing experimental infection with *H. pylori* in animals. Several experimental animal models, such as gnotobiotic piglets (Eaton *et al.*, 1992), C57/BL6 mice (Kim *et al.*, 2008) and Mongolian gerbils, are helpful in understanding the pathogenesis of human *H. pylori* infection. The Mongolian gerbil model, in which *H. pylori* is able to colonize long-term, is particularly easy to handle. It is useful as a small-animal model for the severe inflammation and obvious ulceration caused by *H. pylori* (Matsumoto *et al.*, 1997; Hirayama *et al.*, 2002). *H. pylori* is inhibited by a number of commensal bacterial species as well as opportunistic human pathogens (Krausse *et al.*, 2005). Probiotics including live bacterial cells can also improve the intestinal microflora and modulate immune functions in beneficial ways (Gill & Guarner, 2004; Borchers *et al.*, 2009). Probiotics have been shown to function as antimicrobial effectors (Cross, 2002). Oral administration of certain lactic acid bacteria can prevent pathogenic infection by microbes such as *Listeria monocytogenes* (Popova *et al.*, 1993), *Escherichia coli* (Ishida-Fujii *et al.*, 2007), *Klebsiella pneumoniae* (Gonchar *et al.*, 2009) and *Salmonella* serotype Enteritidis (Jain *et al.*, 2009) through the regulation of inflammatory cytokines. Although the immunomodulatory effects of fermented products have been reported elsewhere (Michetti *et al.*, 1999; Halper *et al.*, 2003; Kim *et al.*, 2008; Kato-Mori *et al.*, 2010), the mechanism by which cell-free products, i.e. fermentation metabolites, stimulate the immune system remains poorly understood. In addition, these probiotic bacteria can be used to supplement eradication therapy for patients with *H. pylori* infection, either to increase the eradication rate or to prevent the occurrence of side-effects of antimicrobial drugs (International Agency for Research on Cancer, 1994; Ferrero & Fox, 2001). Based on the results of those studies, it is possible that gastric bacteria might affect the colonization of *H. pylori* in the gastric mucosa.

In our previous study (Zaman *et al.*, 2010), *Lactobacillus* spp. were isolated from the gastric mucosa of a gerbil

uninfected with *H. pylori*. The uninfectivity of the gerbil was detected on the basis of the least frequency of detection of *H. pylori ureA* in the faecal sample. The presence of *Lactobacillus* spp. in the gastric mucosa of that uninfected gerbil represents the possibility of an inhibitory effect by this species of gastric bacteria on colonization by *H. pylori*. It was suspected that some gastric bacteria may inhibit persistent infection of *H. pylori*, and thus the gastric bacterial microflora isolated from the gastric mucosa were analysed and compared.

In the present study, the gastric microflora of Mongolian gerbils was analysed by 16S rRNA gene sequencing after inoculating the gerbils with the gastric mucosa samples of *H. pylori*-positive and -negative gerbils, dividing them into two separate groups. All of the gerbils were challenged with *H. pylori*. It was also noted that further study to examine the direct effect of the isolated *Lactobacillus* strains separately or combined is necessary.

METHODS

Animals. We purchased 5-week-old female Mongolian gerbils (MGS/Sea; specific-pathogen-free; body weight 25–35 g) from Kyudou, and bred them under specific-pathogen-free conditions in plastic cages and under standard laboratory conditions (room temperature 23±2 °C; relative humidity 40–60 %; 12 h light/dark cycle) in the animal facility of Kyorin University. Standard rodent food pellets (CE-2; Clea Japan) and sterilized tap water were provided *ad libitum* in micro-isolator units as described previously (Krausse *et al.*, 2005). The experiments were approved by the Experimental Animal Ethics Committee of Kyorin University School of Medicine on 1 April 2008 (approval no. 75).

Bacterial strain and culture. *H. pylori* strain TK1402 was isolated from gastric biopsy specimens of patients with gastric and duodenal ulcers (Osaki *et al.*, 1998, 2006). This strain exhibits infectivity in germ-free mice (Osaki *et al.*, 1998), C57/BL6 mice (Yamaguchi *et al.*, 2003) and Mongolian gerbils (Nakagawa *et al.*, 2005). The TK1402 strain was cultured for 2 days in *Brucella* Broth containing 1.5 % agar (Difco) and 7 % horse serum (SBHS-agar; Sigma) under microaerobic conditions at 37 °C using Anaero Pack (A28; Mitsubishi Gas Chemical) containing 85 % N_2, 10 % CO_2 and 5 % O_2.

Animal experiments. The stocked gastric mucosa samples of the *H. pylori*-negative and -positive gerbils of our previous experiment were inoculated into another five and six Mongolian gerbils (5 weeks old) in Groups 1 and 2, respectively. One week after this inoculation, all of the gerbils were inoculated with *H. pylori* TK1402 (1×10^9 c.f.u.). *H. pylori* TK1402 was harvested in Hanks' balanced salts solution (HBSS; Sigma) after incubation and 1 ml aliquots from the prepared bacterial suspension containing 1×10^9 c.f.u. were used. All gerbils were sacrificed 4 weeks after the inoculation of *H. pylori* and gastric mucosa samples were collected. The mucus layer of the stomach was scraped off with a spatula, collected into 500 µl HBSS and homogenized for determination of the number of micro-organisms in the mucus layer (mucosa). *H. pylori*-selective medium (Nissui Pharmaceutical) was inoculated with 50 µl of the gastric sample and incubated at 37 °C for 5 days for the identification of *H. pylori*. Purple colonies were counted and the number of viable *H. pylori* cells was expressed as c.f.u. g^{-1} of the gastric mucosa. *Brucella* Agar medium supplemented with 7 % horse serum was inoculated with a single colony for identification of the bacteria. The isolated strain was shown to be positive for urease, catalase and oxidase with a Gram-negative helical form, and was thus identified as *H. pylori*. All the gastric mucosa samples were used for culture and also stocked at −80 °C for future experiments, such as identification of gastric flora and PCR examination.

Isolation of *H. pylori* from the gastric mucosa. *H. pylori* was isolated from the gastric mucosa samples of the two separate groups of Mongolian gerbils (Groups 1 and 2) after killing the gerbils at 4 weeks post-inoculation with *H. pylori* TK1402. Group 1 gerbils were inoculated with gastric mucosa samples of *H. pylori*-positive gerbils and Group 2 gerbils were inoculated with gastric mucosa samples of *H. pylori*-negative gerbils (Zaman *et al.*, 2010).

DNA extraction from gastric mucosa samples. For DNA extraction, 200 µl gastric mucus sample suspension was added to a solution containing 250 µl extraction buffer (200 mM Tris/HCl, 80 mM EDTA; pH 9.0) and 50 µl of 10 % SDS. Then, 300 mg of glass beads (diameter 0.1 mm) and 500 µl buffer-saturated phenol were added to the suspension, and the mixture was vortexed vigorously at 4200 r.p.m. for 30 s using a Mini Bead Beader (Wakenyaku). After centrifugation at 14 000 ***g*** for 5 min, 400 µl supernatant was collected, phenol/chloroform extractions were performed and 250 µl supernatant was subjected to 2-propanol precipitation. Finally, the DNA was suspended in 1 ml Tris/EDTA buffer.

Total DNA (1 µl) was amplified using PCR primers for the 16S rRNA of *H. pylori*, HP-16-F (5′-CGCTAAGAGATCAGCCTATGTCC-3′) and HP-16-R (5′-CCGTGTCTCAGTTCCAGTGTGT-3′), for the detection of *H. pylori* (Osaki *et al.*, 2006), and g-Lact-F (5′-ACCACAGTCCATGCCATCAC-3′) and g-Lact-R (5′-TCCACCACCCTGTTGCTGTA-3′) primers for the detection of lactobacilli (Rinttilä *et al.*, 2004).

Quantitative real-time PCR. A real-time PCR assay was performed using the method reported by Rinttilä *et al.* (2004), with some modifications. Quantitative analysis was performed using SYBR Green methods. Each reaction mixture (10 µl) was composed of 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 µM, a 1:75 000 dilution of SYBR Green I, 11 ng *Taq* Start antibody (Clontech) μl^{-1}, 0.005 U *Taq* DNA polymerase (Takara) μl^{-1}, each of the specific primers at a concentration of 0.25 µM and 1 µl of × 1 or × 10 diluted template DNA. The amplification program consisted of one cycle at 94 °C for 5 min, followed by 45 cycles at 94 °C for 20 s, 60 °C for 20 s and 72 °C for 35 s, and finally one cycle at 94 °C for 30 s. We used an absolute quantification in which DNA target genes were compared with data from a standard curve, which was generated by amplifying serial dilutions of a known number of *H. pylori* TK1402 or *Lactobacillus gasseri* YIT 0192^T. For each primer set, PCR was performed in parallel reactions using different amounts of *H. pylori* TK1402 chromosomal DNA. Quantification data were analysed using 7600 quantification software (Applied Biosystems). In this analysis, the background fluorescence was removed by manually setting a noise band. The long-linear portion of the standard amplification curve was identified, and the crossing point was the intersection of the best-fit line through the long-linear region and the noise band. The standard curve was a plot of the crossing points versus the log bacterial number (c.f.u. ml^{-1}). The quantification software determined the unknown concentration by interpolating the noise band intercept of an unknown sample against the standard curve of known concentrations. The quantitative data were calculated from the standard curve of the PCR. For this approach, the identity and specificity of the PCR product were confirmed by dissociation curve analysis, which is part of the 7600 quantification program. To confirm the specificity of the PCR product, a melting curve analysis was performed after amplification to distinguish the targeted PCR product from the

non-targeted PCR product. The melting curves were obtained by slow heating at temperatures from 60 to 95 °C at a rate of 0.2 °C s^{-1}, with continuous fluorescence collection. The presence of lactobacilli among both Groups 1 and 2 was confirmed by performing real-time PCR. The number of lactobacilli quantified by real-time PCR was measured per gram of the gastric mucosa.

Isolation of gastric bacteria and their identification using API 20E and the 16SrRNA gene sequencing system. Gastric bacteria were isolated from the gastric mucosa samples of both Group 1 and Group 2 gerbils. Identification of the isolated gastric bacteria was performed using API 20E and the 16S rRNA gene sequencing method. The 16S rRNA gene sequencing method is more reliable than the API 20A system for the identification of anaerobic bacteria. To confirm the identities of bacterial species, extracted DNA samples after PCR amplification were used for the sequencing of 16S rRNA genes. DNA was extracted from the biopsy samples.

Facultative anaerobic bacteria were isolated from the gastric mucosa samples of the 11 gerbils (1-1–1-5 and 2-1–2-6) by aerobic culture using DHL plates. To isolate the dominant bacterial species from each gerbil, the gastric specimen and 10-fold dilutions of the suspension were inoculated on Gifu anaerobic medium (GAM) agar (Nissui Pharmaceutical), phenyl ethyl alcohol (PEA)-blood agar (Nissui Pharmaceutical) supplemented with 5 % horse blood and DHL agar (Nissui Pharmaceutical). GAM agar and PEA-blood agar were incubated under anaerobic conditions at 37 °C in an anaero incubator (Hirasawa) for 48 h. DHL and PEA-blood agar were aerobically incubated at 37 °C for 24 h. After incubation, it was noticed that the growth of bacteria under the anaerobic conditions was more prominent than under the aerobic conditions. Each different type of colony was inoculated for single-colony isolation, and incubated under aerobic and anaerobic conditions at 37 °C. The facultative anaerobic isolates were identified using the API 20E system (bioMérieux), whereas the obligate anaerobes were identified using the genomic sequencing method. These obligate anaerobes isolated from the Mongolian gerbil gastric mucosa samples were cultured in Man–Rogosa–Sharpe (MRS) broth or agar (Difco), and incubated under anaerobic conditions in an atmosphere of 80 % N_2, 10 % H_2 and 10 % CO_2 at 37 °C. Genomic DNA from the cultured bacteria of the gastric mucosa samples was extracted using a MagExtractor (Toyobo).

PCR was performed in thin-walled 0.5 ml Gene Amp reaction tubes (PerkinElmer). Aliquots of 1 µl extracted genomic DNA were mixed with each primer solution (5 pmol) and 0.5 U *Taq* polymerase in a total volume of 20 µl. Reactions were performed for 30 cycles of 94 °C for 5 min, 94 °C for 1 min, 61 °C for 20 s and 72 °C for 1 min with a thermal cycler (Gene Amp PCR system 9600-R; PerkinElmer). We used two universal primers (27F, 5′-AGAGTTTGATCMTGGC-TCAG-3′; 1492R, 5′-TACGGYTACCTTGTTACGACTT-3′) (DeLong, 1992) and two primers (518F, 5′-CCAGCAGCCGCGGTAATACG-3′; 800R, 5′-TACCAGGGTATCTAATCC-3′) (Stackebrandt & Goodfellow, 1991) for PCR. After the PCR products were obtained, the mixture was purified using a PCR clean-up system (Promega) for the sequencing study. Sequencing reactions were performed in a Bio-Rad DNA Engine Dyad PTC-220 Peltier Thermal Cycler using ABI BigDye Terminator v3.1 Cycle Sequencing kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using universal primers (518F and 800R). The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The sequencing of each PCR was analysed by Sequence Scanner (ABI). A BLAST search was performed using Lasergene 7 (DNASTAR).

Preparation of culture filtrates of lactobacilli. Lactobacilli cultures were maintained as frozen stocks held at −80 °C in MRS broth (Difco) plus 20 % glycerine (Fisher Scientific). Lactobacilli cultures were propagated and transferred once before use. MRS agar was prepared by the addition of 1.5 % (w/v) granulated agar (BBL Microbiology Systems) to the broth medium. Lactobacilli were inoculated on MRS agar and incubated under anaerobic conditions at 37 °C in an anaero incubator (Hirasawa) for 48 h. Liquid culture of lactobacilli was performed using MRS broth (Oxoid) at 37 °C in 5 % CO_2 for another 2 days. After centrifugation of the liquid culture broth of the lactobacilli for 48 h, culture filtrates were collected and preserved at −20 °C for performing the growth inhibition assay of *H. pylori*.

As *H. pylori* was not detected in gerbil 1-2 and gerbil 1-5, and their gastric mucosa samples inhibited the growth of *H. pylori*, two lactobacilli strains (*Lactobacillus reuteri* and *Lactobacillus johnsonii*) were chosen from lactobacilli isolated from gerbil 1-2 and three different lactobacilli strains (*L. reuteri*, *Lactobacillus murinus* and *L. johnsonii*) were chosen from gerbil 1-5 as representative strains with inhibitory activity against *H. pylori*.

Growth inhibition of *H. pylori* TK1402 using brain heart infusion (BHI) broth culture filtrates of gastric mucosa samples of Group 1 gerbils. The growth-inhibitory effect on *H. pylori* TK1402 was analysed using BHI broth culture filtrates of the gastric mucosa samples of the gerbils in Group 1. *H. pylori* TK1402 was cultured overnight and 32-fold dilution of BHI broth culture filtrates of gastric mucosa samples of Group 1 gerbils was prepared with 7 % horse serum. An aliquot of 50 µl of suspension of the precultured *H. pylori* was inoculated in 100 µl of diluted samples into 96-well plates after setting the final OD_{595} as 0.05. Shaking culture using the culture filtrates of the *H. pylori*-negative gerbils was done for 48 h. After the incubation, OD_{595} of each sample was measured by a microplate reader (Mithras LB940; Berthold Technologies). Growth of *H. pylori* in BHI broth supplemented with 7 % horse serum was used as control.

Growth inhibition of *H. pylori* TK1402 using MRS broth culture filtrates of lactobacilli strains. *H. pylori* TK1402 was propagated and transferred once before use. *Brucella* Agar medium supplemented with 7 % horse serum was used for the culture of *H. pylori* TK1402 at 37 °C. The lactobacilli strains were isolated from gastric mucosa samples of gerbil 1-5 of Group 1 and gerbil 2-2 of Group 2. Various dilutions of the MRS broth culture filtrates of five lactobacilli were prepared with *Brucella* Broth containing 7 % horse serum. MRS broth was used as control to compare the inhibitory effect of this broth and it was also diluted with *Brucella* Broth containing 7 % horse serum. An aliquot of 50 µl of suspension of the precultured *H. pylori* was inoculated in 100 µl of various diluted samples into 96-well plates after setting the final OD_{595} as 0.050. Shaking culture using the culture filtrates of five lactobacilli was performed for 48 h. After incubation, OD_{595} of each sample was measured by a microplate reader.

Statistical analysis. The data were analysed using StatView software. Group differences were tested with Student's *t*-test or the χ^2 test. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Quantification of *H. pylori* and lactobacilli by real-time PCR and isolation of gastric bacteria

In the gastric mucosa samples of the Group 1 gerbils, no *H. pylori* was observed in three gerbils (1-2, 1-4 and 1-5), although two gerbils (1-1 and 1-3) showed the presence of

Table 1. Detection of *H. pylori* in Group 1 and Group 2 gerbils

	Group 1					Group 2					
	1-1	1-2	1-3	1-4	1-5	2-1	2-2	2-3	2-4	2-5	2-6
Body weight (g)	52.8	59.3	53.0	58.5	52.9	64.5	59.0	62.0	63.5	63.5	58.5
Stomach weight (g)	1.9	2.0	1.9	1.7	1.8	2.8	2.3	2.7	2.6	2.3	2.5
Gastric pH	2.5	3.0	3.0	3.0	3.0	3.0	3.0	4.5	4.5	4.5	3.0
No. (log_{10}) *H. pylori* (real-time PCR)	5.31	ND	4.31	ND	ND	ND	9.56	4.42	4.54	ND	4.79
No. (log_{10}) of *Lactobacillus* spp. g^{-1} mucus (real-time PCR)	10.58	12.18	10.76	ND	10.80	8.78	10.65	11.40	10.97	11.18	9.77

ND, Not detected.

H. pylori by real-time PCR (Table 1). The presence of *H. pylori* was observed in four (2-2, 2-3, 2-4 and 2-6) of six gerbils in Group 2 inoculated with the gastric mucosa samples of *H. pylori*-positive gerbils, but another two gerbils (2-1 and 2-5) showed no colonization by *H. pylori*. There was no significant difference in the number of *H. pylori* as determined by real-time PCR between Groups 1 and 2. The colonization rate of *H. pylori* in Group 1 gerbils (2/5, 40 %) was lower than that in Group 2 gerbils (4/6, 67 %).

For the inoculation of gerbils, *H. pylori* TK1402 was selected as described previously (Nakagawa *et al.*, 2005). In this study, we used Mongolian gerbils to analyse the microflora in the stomach of the gerbil infected with *H. pylori* previously (Zaman *et al.*, 2010). Marchetti *et al.* (1995) reported the successful colonization by clinical isolates of *H. pylori* strains, but not NCTC 11637 (an established laboratory strain), in conventional and specific-pathogen-free mice. Oral administration of *H. pylori* TK1402 induced colonization and gastric inflammation of the stomach of Mongolian gerbils (Nakagawa *et al.*, 2005). The difficulty of colonization in experimental animals such as piglets and mice by *H. pylori*, except when maintained in germ-free and decontaminated conditions, has been reported previously (Krakowka *et al.*, 1987; Ohnishi, 1996).

There was no significant difference in the number of lactobacilli between Group 1 (1-1, 1-2, 1-3 and 1-5) and Group 2 (2-1–2-6) gerbils (Table 1). This result shows *Lactobacillus* spp. to be the dominant bacteria in the stomach of Mongolian gerbils as the presence of lactobacilli was observed in both groups of gerbils. The difficulty of colonization by *H. pylori* in conventional mice may be explained by the large number of indigenous lactobacilli in their stomachs (Kabir *et al.*, 1997). However, a very small number of lactobacilli inhabiting the stomachs of humans may permit *H. pylori* to colonize this organ easily.

Lactobacilli are components of the normal intestinal flora of healthy humans that exert antagonistic activities against pathogens. The reason for the presence of lactobacilli in both of the two groups of gerbils is not clear yet. The composition of the gastric microflora, including lactobacilli, may have an influence on the colonization of *H. pylori*. In particular, it is known that the primary microorganisms associated with the stomach belong to the genus *Lactobacillus*. *Lactobacillus* shows a particular capacity to survive and develop in an acidic environment, and can live as an indigenous bacterium in the gastric mucosa, which can effectively inhibit the colonization of *H. pylori* (Peek, 2008; Chen *et al.*, 2012). In other words, it is rational to prevent and control *H. pylori* infection by regulating the balance of the flora in the stomach. Thus, *Lactobacillus* can be a choice to replace antibiotics or as an adjuvant to antibiotics in treating *H. pylori* infection.

Stomach weights and gastric pH of Mongolian gerbils after inoculation with the gastric mucosa samples

Although there was some variation of the pH, there was no significant difference in the pH between the two groups (Fig. 1a). The stomach weight of Group 1 gerbils was significantly lower than that of Group 2 gerbils (Fig. 1b). Although the reason for the difference is unclear, it is possible that colonization of *H. pylori* in the gastric mucosa induced various inflammatory changes, including cell

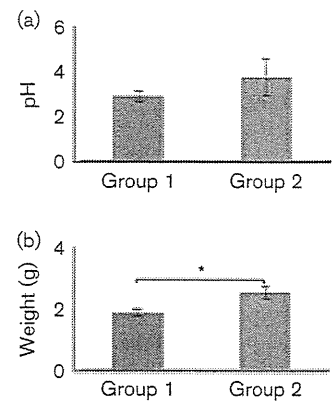

Fig. 1. Measurement of (a) pH and (b) stomach weight of Group 1 and 2 Mongolian gerbils after inoculation with gastric mucosa samples.

Table 2. Identification of facultative anaerobes isolated by aerobic culture from the gastric mucosa samples of Mongolian gerbils

Group 1					Group 2					
1-1	1-2	1-3	1-4	1-5	2-1	2-2	2-3	2-4	2-5	2-6
E. coli *Kluyvera* spp.	*E. coli* *Kluyvera* spp.	*E. coli*	*E. coli* *Kluyvera* spp.	*E. coli* *Kluyvera* spp.	*E. coli*	*E. coli* *Kluyvera* spp.	*E. coli* *Kluyvera* spp.	*E. coli* *Kluyvera* spp.	*Kluyvera* spp.	*E. coli* *Kluyvera* spp.

filtration (Mishra & Panigrahi, 2011) and edematous changes, resulting in the weight variations.

Identification of different gastric bacteria after isolation from the gastric mucosa samples

Isolated facultative anaerobic bacteria were identified by API 20E (Table 2). *E. coli* were isolated from 10 out of 11 gerbils (except gerbil 2-5) and *Kluyvera* spp. were isolated from nine out of 11 gerbils (except gerbils 1-3 and 2-1).

Using anaerobic cultivation of the gastric mucosa of the 11 gerbils, bacterial colonies were formed from the gastric mucosa samples of all of the gerbils (Table 3).

Three species of lactobacilli were isolated from the gastric mucosa samples, and they were identified by genome sequencing as *L. murinus*, *L. reuteri* and *L. johnsonii*. Table 3 shows three different strains of lactobacilli (*L. murinus*, *L. reuteri* and *L. johnsonii*) in Group 1 gerbils and two strains of lactobacilli (*L. reuteri* and *L. johnsonii*) in Group 2 gerbils. Some of the bacterial strains could not be to be determined (shown as undetermined in Table 3). Although *L. reuteri* and *L. johnsonii* strains were isolated in all Group 1 and Group 2 gerbils, *L. murinus* was present only in gerbil 1-5. According to the genome sequencing, *L. reuteri* was grouped into two subtypes: LR1 and LR2 (Fig. 2). There were several differences in base sequences in the 16S rRNA gene between LR1 and LR2 subtypes of *L. reuteri*. In contrast, there was no difference in the 16S rRNA gene sequence of *L. johnsonii* strains. Sequencing of 16S rRNA genes that were PCR-amplified from DNA extracted from the biopsy samples was performed to confirm the identities of different bacterial species.

The bacterial species isolated differed between the previous study (*Actinomyces* spp. or *Bifidobacterium* spp.) and the present study. The reason for the difference is not clear, but the following two possibilities are suggested. (1) The identification method was different; we used the API system previously, but real-time PCR in this study. It is well known that the gene sequencing method is more reliable than the API system for the identification of bacteria. We have already obtained a result where the three strains of *L. reuteri*, *L. johnsonii* and *L. murinus* identified by genome sequencing were identified as *Actinomyces* spp. or *Bifidobacterium* spp. in the API system (data not shown). (2) Inoculation with the gastric mucosa samples may eliminate gastric bacteria other than lactobacilli in our present study.

BHI broth culture filtrates of gastric mucosa samples of Group 1 gerbils exhibit growth inhibition of *H. pylori*

On the basis of the results of the real-time PCR (Table 1), gerbils 1-2, 1-4 and 1-5 were determined as *H. pylori*-

Table 3. Identification of different lactobacilli isolated by anaerobic culture from the gastric mucosa samples of Mongolian gerbils

Group	Gerbil	Isolated lactobacilli			
Group 1	1-1	*L. reuteri*1*,‡	*L. johnsonii*	Undetermined	–
	1-2	*L. reuteri*1	*L. johnsonii*	–	–
	1-3	*L. reuteri*1	*L. johnsonii*	–	*L. reuteri*2†,‡
	1-4	*L. reuteri*1	*L. johnsonii*	Undetermined	*L. reuteri*2
	1-5	*L. reuteri*1	*L. johnsonii*	Undetermined	*L. murinus*
Group 2	2-1	*L. reuteri*1	*L. johnsonii*	Undetermined	–
	2-2	–	*L. johnsonii*	Undetermined	*L. reuteri*2
	2-3	–	*L. johnsonii*	Undetermined	*L. reuteri*2
	2-4	*L. reuteri*1	*L. johnsonii*	Undetermined	–
	2-5	–	*L. johnsonii*	*Lactobacillus* spp.	*L. reuteri*2
	2-6	*L. reuteri*1	*L. johnsonii*	–	–

*Subtype 1 according to genomic sequence.
†Subtype 2 according to genomic sequence.
‡Difference in genomic sequence was detected between *L. reuteri*1 and *L. reuteri*2.

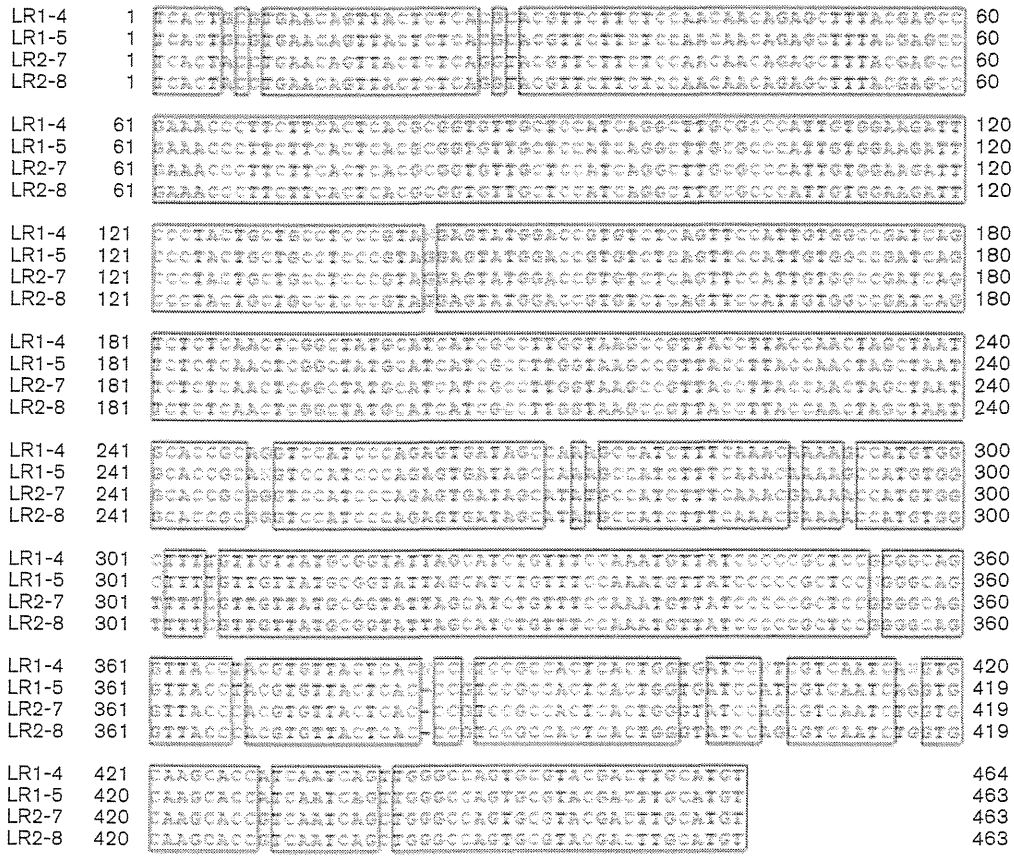

Fig. 2. Sequencing analysis of *L. reuteri*1 (LR1) and *L. reuteri*2 (LR2) isolated from different Mongolian gerbils. LR1-4, LR1 isolated from gerbil 1-4; LR1-5, LR1 isolated from gerbil 1-5; LR2-7, LR2 isolated from gerbil 2-2; LR2-8, LR2 isolated from gerbil 2-3.

negative among the five gerbils in Group 1. As the colonization rate of *H. pylori* in gerbils 1-1 and 1-3 pretreated with *H. pylori*-negative gastric mucosa samples was relatively low, the direct effect of the gastric mucosa samples of the five gerbils of Group 1 on the growth of *H. pylori* was examined. It is possible that the gastric mucosa samples may have an inhibitory effect on the growth of *H. pylori*. It was shown that the culture filtrates (1 : 32 dilution) of two gerbils (1-2 and 1-5) inhibited significantly the growth of *H. pylori* TK1402 (Fig. 3). In particular, it was indicated that the gastric mucosa sample of gerbil 1-5 exhibited the strongest inhibitory effect on the growth of *H. pylori*; the gastric mucosa sample of gerbil 1-2 also exhibited a strong inhibitory effect.

Inhibition of growth of *H. pylori* TK1402 by culture filtrates of five lactobacilli strains

The inhibitory effects of the five lactobacilli strains were clarified through the use of the growth inhibition assay using *H. pylori* TK1402 with MRS broth culture filtrates of these lactobacilli with 7 % horse serum (Fig. 4). The growth of *H. pylori* co-cultured with the culture filtrates of five

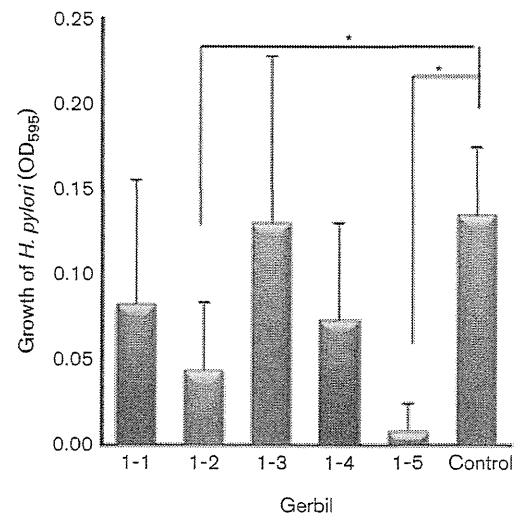

Fig. 3. Growth inhibition of *H. pylori* by culture filtrates (BHI broth) of Group 1 Mongolian gerbils.

lactobacilli was inhibited compared with the control (MRS broth only) on the basis of the inhibition of bacterial growth (OD_{595}). The substances of the culture filtrates of lactobacilli may have a suppressive effect on the growth of *H. pylori*. The inhospitable acidic milieu in the stomach provides an effective barrier, killing many of the microbes that enter the gastrointestinal tract. It was shown in various studies that *Lactobacillus* spp. were the dominant bacteria in the stomach of *H. pylori*-infected, as well as control, gerbils (Osaki *et al.*, 2012; Sun *et al.*, 2003). *L. gasseri* and *L. reuteri*, which are present in the stomach of most Mongolian gerbils, were also shown to inhibit the growth of some *H. pylori* strains. *L. gasseri* OLL 2716 promoted the elimination of *H. pylori* in humans (Johnson-Henry *et al.*, 2004) and exerted a protective effect against the generation of lesions in a rat gastric ulcer model. In addition, these probiotic bacteria can be used to supplement eradication therapy for patients with *H. pylori* infection, either to increase the eradication rate or to prevent the occurrence of the side-effects of the antimicrobial drugs (Mégraud, 2004), and are utilized in yogurts that are specifically labelled as health foods (Wang *et al.*, 2004).

Eradication therapy of *H. pylori* infection by triple association of two antibiotics and a proton pump inhibitor has been reported. However, the treatment may fail in 10–35 % of cases due to *H. pylori* resistance, antibiotic side-effects and other reasons (Tursi *et al.*, 2004). The search for new or additional therapeutic agents is necessary to overcome treatment failure. Probiotic bacteria are defined as commensals that, when administered to humans, have an inherent benefit over and above nutrition (Guarner & Schaafsma, 1998). Some probiotics, including lactobacilli, have been shown previously to decrease inflammatory markers in *H. pylori* infection models both *in vitro* and *in vivo* (Johnson-Henry *et al.*, 2004; Tamura *et al.*, 2006).

We studied the microbial ecology in the stomach of Mongolian gerbils with *H. pylori* infection. Observations *in*

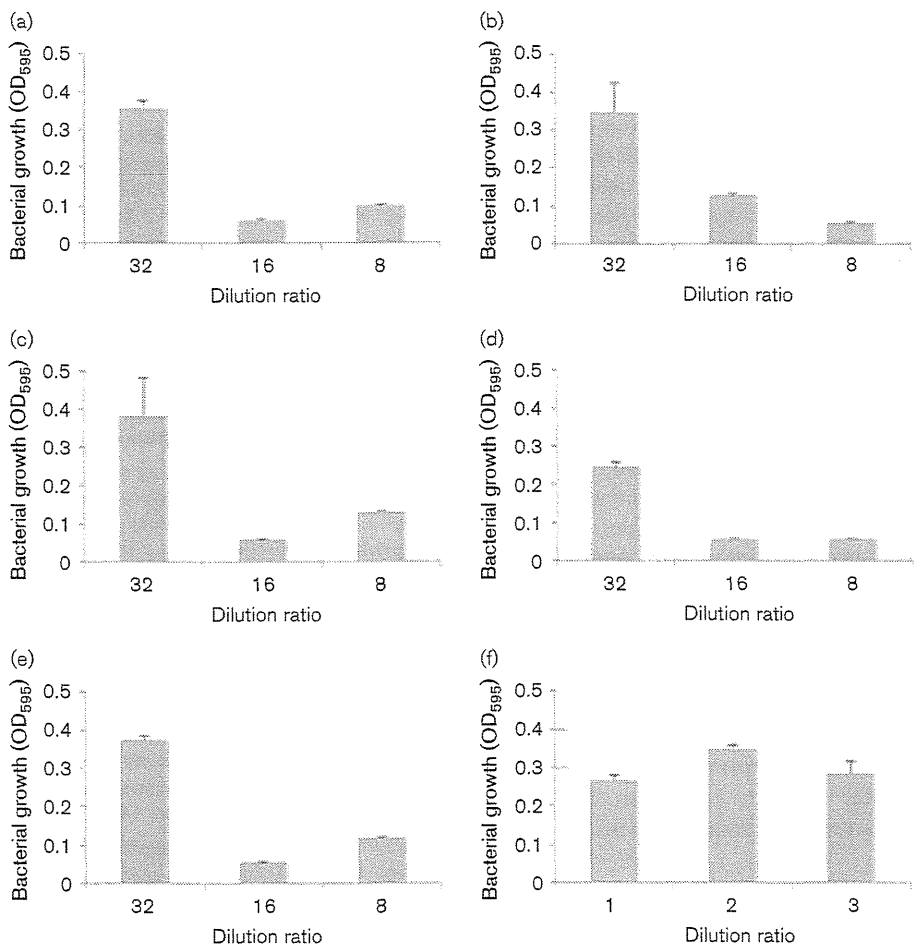

Fig. 4. Growth inhibition assay of *H. pylori* TK1402 by culture filtrates (MRS broth) of five lactobacilli at various dilutions: (a) *L. murinus* isolated from gerbil 1-5, (b) *L. reuteri* isolated from gerbil 1-5, (c) *L. johnsonii* isolated from gerbil 1-5, (d) *L. reuteri* isolated from gerbil 1-2, (e) *L. johnsonii* isolated from gerbil 1-2 and (f) control (MRS broth).

vitro indicated that spent supernatant of *L. acidophilus* La1 and *L. reuteri* contained a bactericidal activity effective on *H. pylori* (Michetti *et al.*, 1999; Ojetti *et al.*, 2012). Non-pathogenic lactobacilli have been used for decades because of their health benefits and their ability to increase resistance to infectious illness (Gill *et al.*, 2000; Chen *et al.*, 2012). Eradication of *H. pylori* was recently shown to decrease the incidence of gastric cancer (Tatematsu *et al.*, 2007; Fukase *et al.*, 2008). These studies suggest that different lactobacilli might be effective in producing a suppressive activity against *H. pylori*. Further studies found that lactobacilli can also have a beneficial effect in conjunction with standard antibiotic based triple therapies against *H. pylori*. Future research is required to clarify the suppressive effect of these microflora against *H. pylori* colonization.

CONCLUSIONS

On the basis of the result of the real-time PCR measurements of the number of lactobacilli per gram of gastric mucosa samples, the presence of lactobacilli was observed in almost all of the gerbils, except in one gerbil, although there was no significant difference in the number of lactobacilli between the two groups (Groups 1 and 2) used in this study. Some strains of lactobacilli can colonize the gastric mucosa and exhibit anti-*H. pylori* colonization. Due to differences in species and specificity, these strains can lead to different anti-*H. pylori* activities. Although different strains of lactobacilli, such as *L. murinus*, *L. reuteri* and *L. johnsonii*, were isolated from the Mongolian gerbil gastric mucosa samples, the three strains did not display the same anti-*H. pylori* activity.

The results of the present study suggest that lactobacilli are the dominant gastric microflora of Mongolian gerbils and three different lactobacilli exerted strong growth-inhibitory effects against *H. pylori* in the *in vitro* study. *Lactobacillus* itself is considered to be a harmless organism in the gastric mucosa when it colonizes the stomach. This work provides bacterial targets for further studies on the direct mutual interaction between *H. pylori* and the Mongolian gerbil's gastric microbiota. Future studies examining the direct effect of *L. murinus*, *L. johnsonii* and *L. reuteri* on the colonization of *H. pylori* in the stomach of Mongolian gerbils are necessary. It would be of great interest to further explore the role of such probiotic strains in the complex regulation of anti-*H. pylori* activities and screen for more efficient potential clinical agents.

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