# Adhesion of probiotic LAB to Caco-2 intestinal model system

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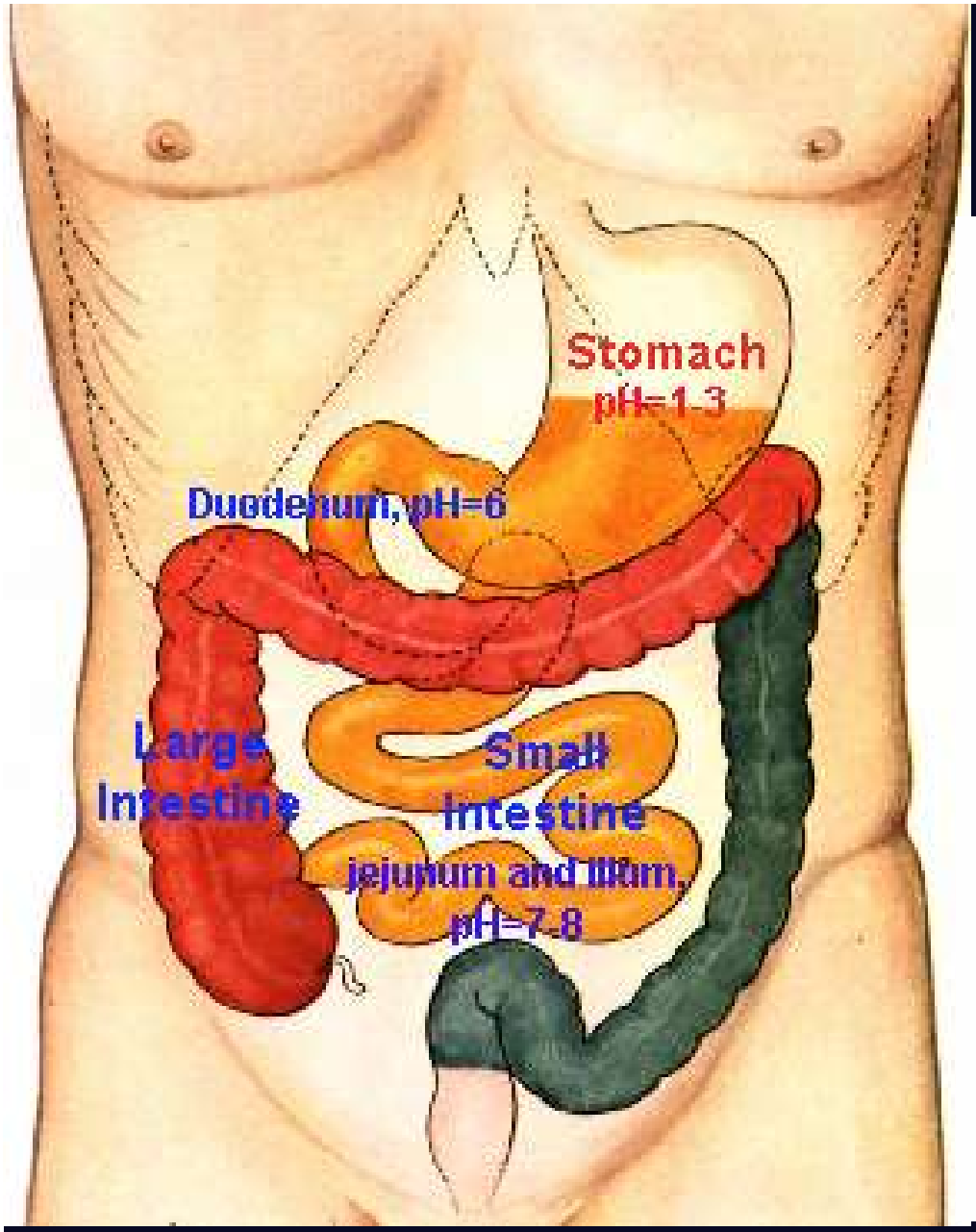
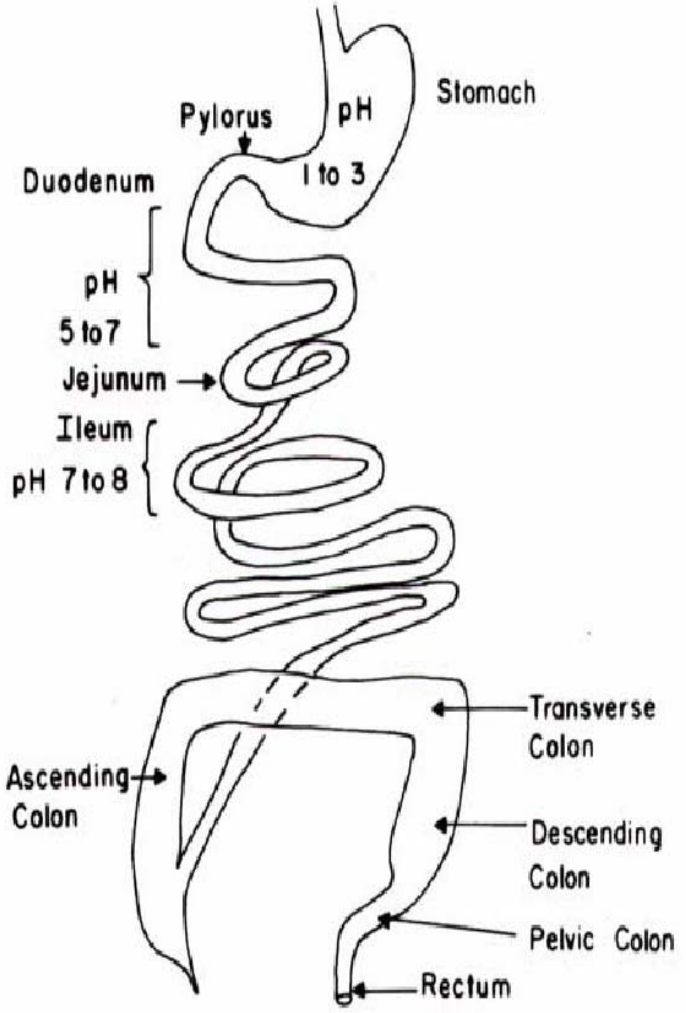
大同大學　生物工程系

2006/11/03


Stomach
pH=1.3
Duodenum, pH=6
Large
Intestine
Small
Intestine
jejunum and ilium,
pH=7.8


Stomach
Pylorus
pH
1 to 3
Duodenum
pH
5 to 7
Jejunum
Ileum
pH 7 to 8
Transverse
Colon
Ascending
Colon
Descending
Colon
Pelvic Colon
Rectum

# Colonic Microorganisms

- Indigenous microorganisms lining the large intestine
- Form a symbiotic relationship with the host
- Provide health benefits
- Protect the host from pathogens
- Control potential pathogens
  - Bacterial antagonism
  - Barrier resistance
  - Colonization resistance

# Colonic Microorganisms

- **Metabolize and detoxify harmful substances**

  **Decreased azoreductase, nitroreductase and glucuronidase activities that are species and strain specific**

- **Stimulate the immune system**

  **Increased gamma interferon (INF$\gamma$) and circulating NK cells**

# Potential Insults to the Microflora

**Starvation**

- **Mucosal atrophy**
- **Decreased protective mucus layer**

**Low fiber diets**

- **Reduced SCFA, the preferred fuel for colonocytes**
- **Reduction in the microflora**

# Potential Insults to the Microflora

**Antibiotics**

- **Reduce total GOOD bacteria and alter the microflora balance**
- **Allow proliferation of potentially harmful bacteria & fungi**
  - *C. difficile*
  - *C. albicans*
  - *S. aureus*
- **Emergence of resistant strains**

# Probiotics

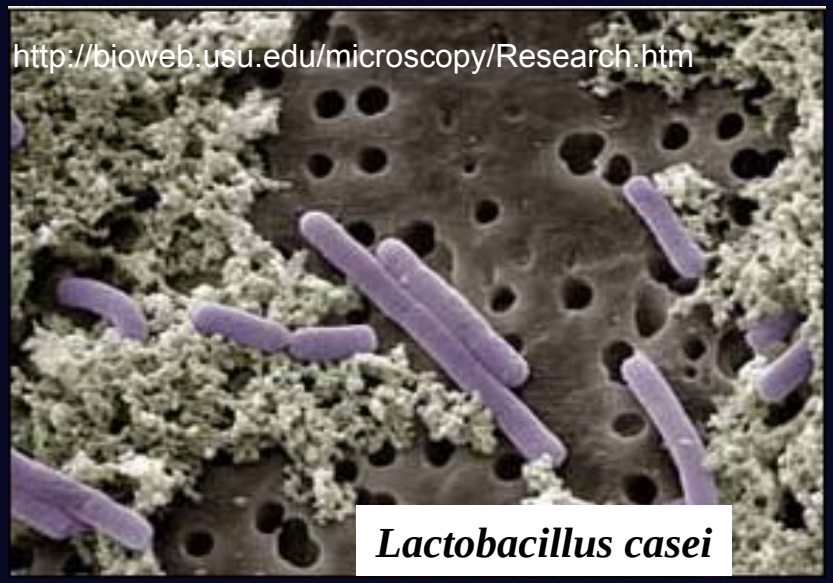
**« Defined, live microorganisms which, administered in adequate amounts, confer a beneficial physiological effect on the host »**

*Source: Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food (2002)*

**« A monoculture or mixed-culture of live microorganisms that benefit the microbiota indigenous to humans »**

*Source: Natural Health Products Regulations P.C. 2003-847 5 June, 2003. 2003-06-18 Canada Gazette Part II, Vol. 137, No. 13*

# 常見乳酸菌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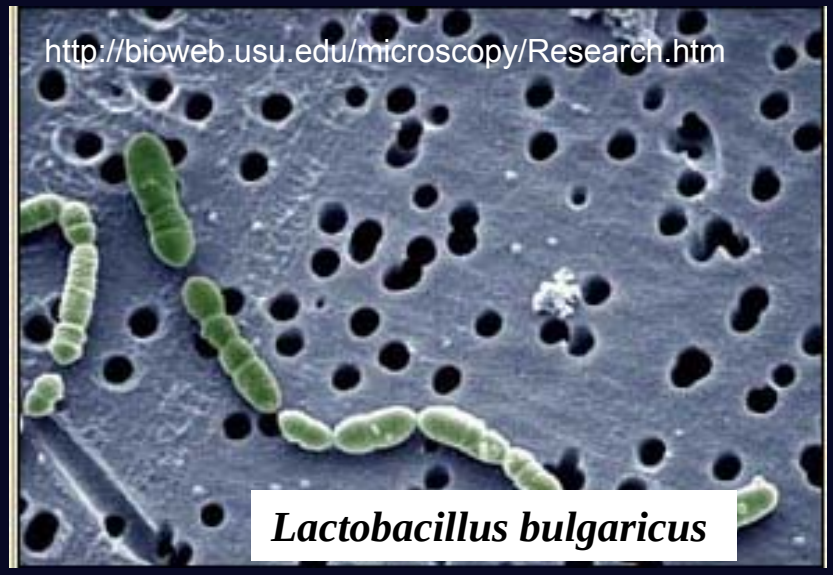
Lactobacillus casei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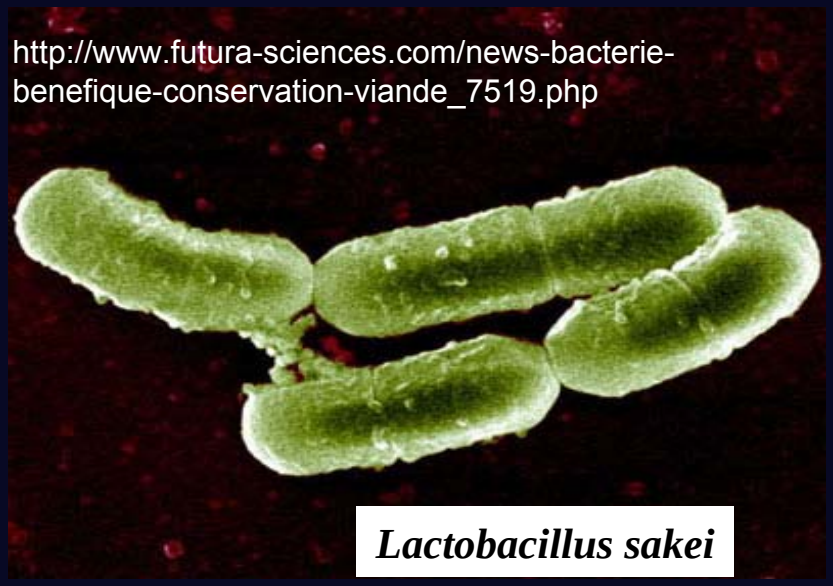
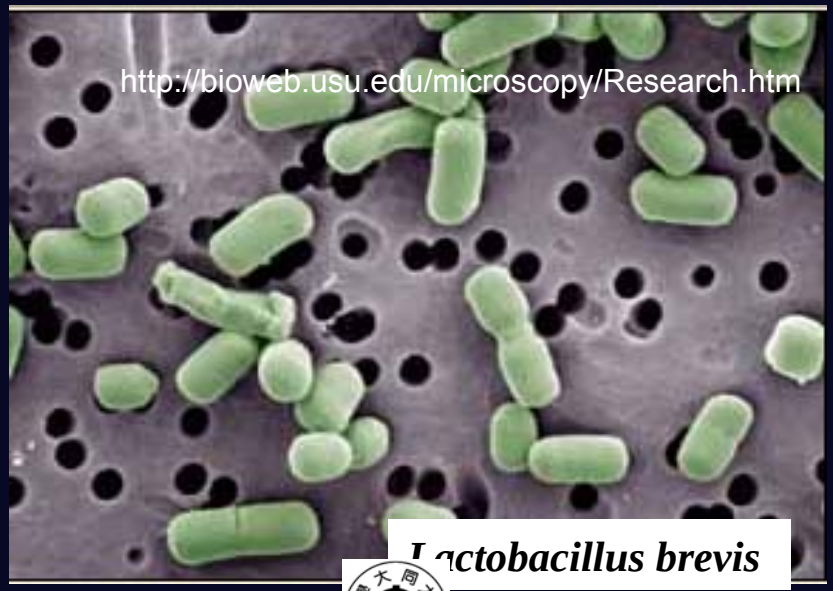
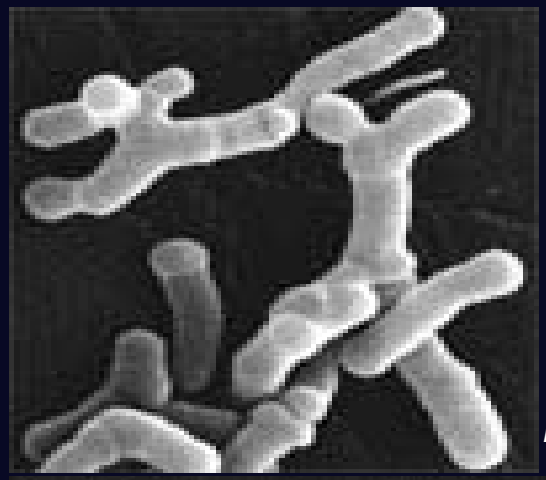
Lactobacillus bulgaricus

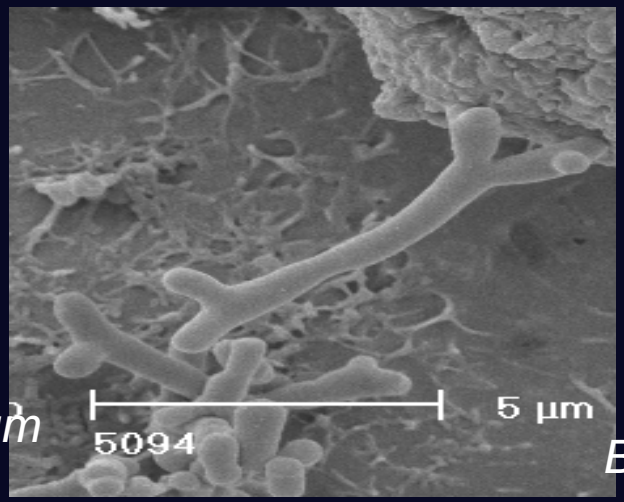

http://www.futura-sciences.com/news-bacterie-benefique-conservation-viande_7519.php
Lactobacillus sakei


http://bioweb.usu.edu/microscopy/Research.htm
Lactobacillus brevis

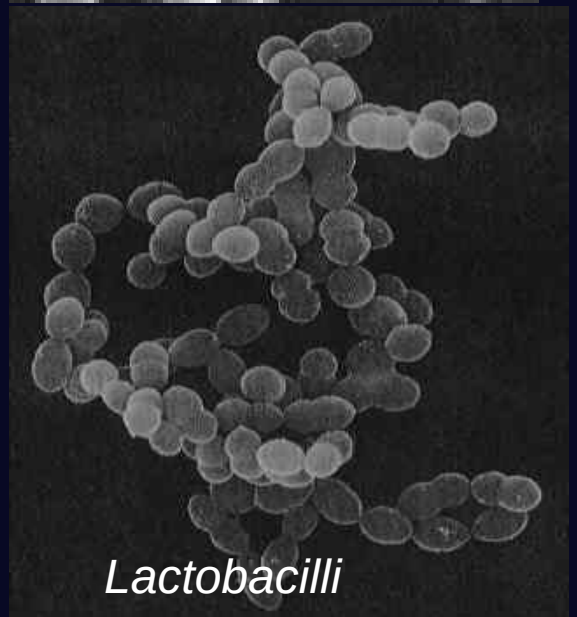
# 常見乳酸菌



*Bifidobacterium*




*Bifidobacterium*



*Lactobacilli*

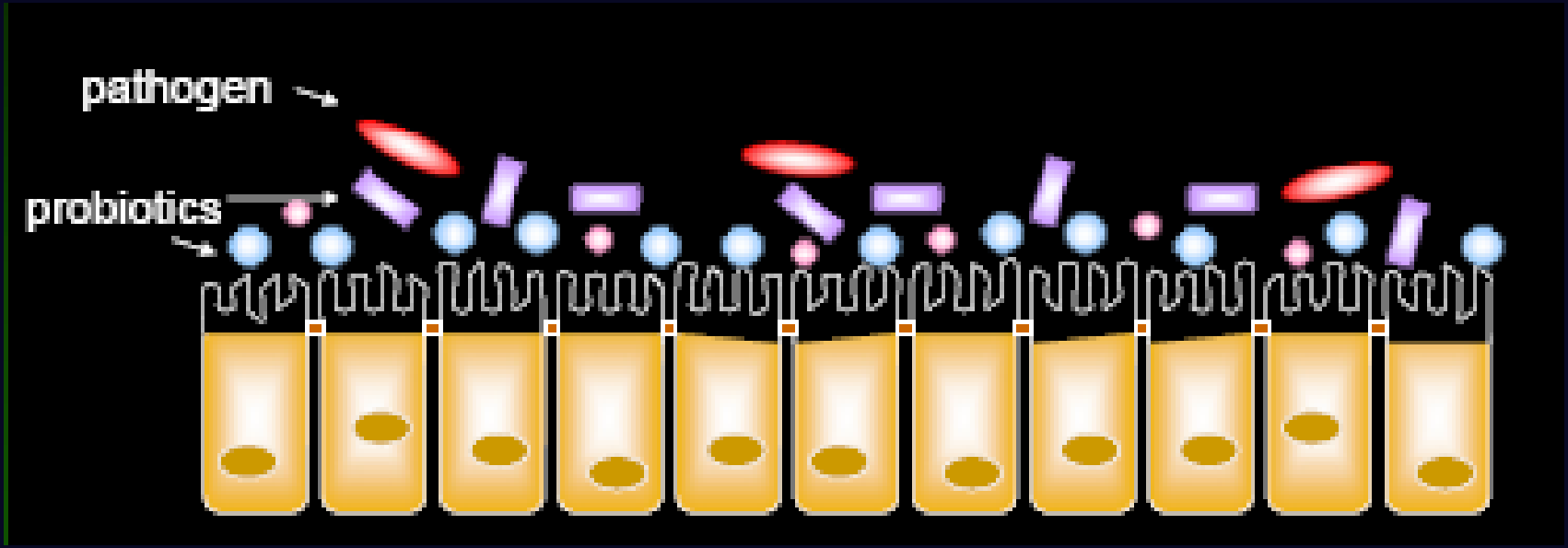
*Streptococci* and *Lactobacilli* in Yogurt

http://homepage1.nifty.com/masa_i/biol03.htm

# Desirable Properties of Probiotics

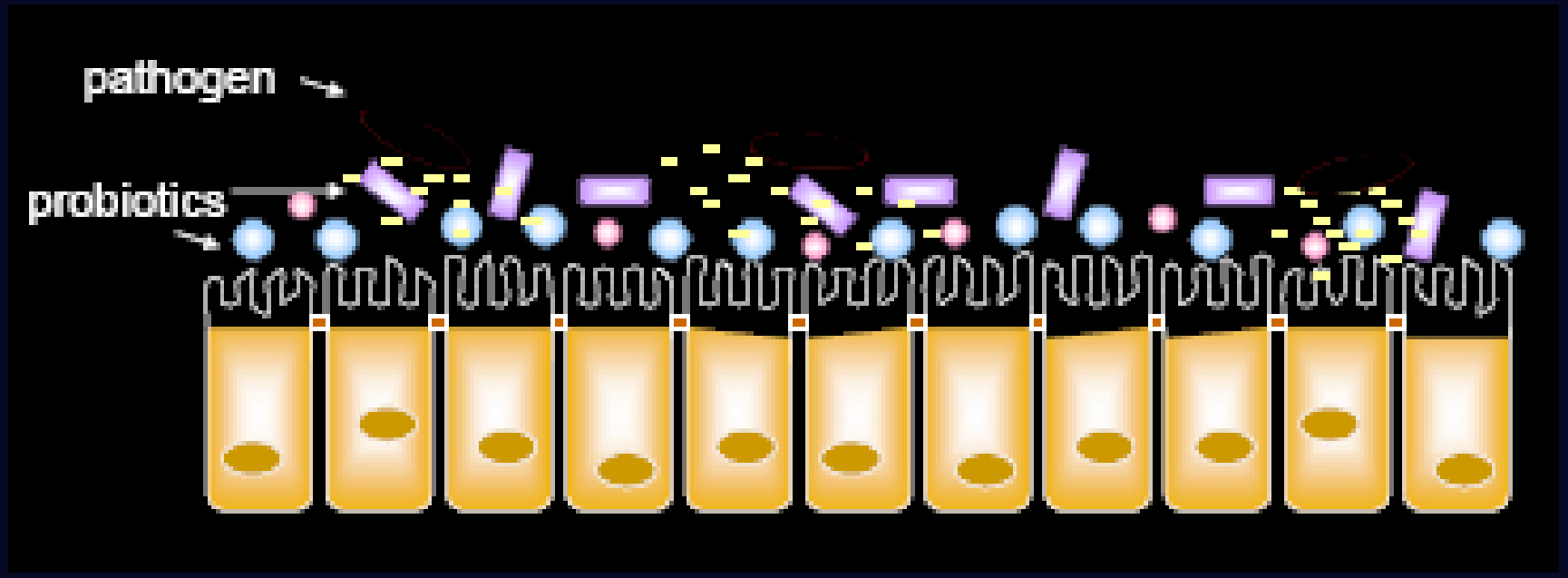
1. Human origin
2. Ability to resist upper GI tract secretions (acid, bile,enzymes)
3. Adherence to human intestinal cells
4. Colonization of the human intestinal tract
5. Production of antimicrobial substances
6. Antagonism against carcinogenic/pathogenic organisms
7. Safety in food and clinical use
8. Clinically-proven health benefits
9. Technologic properties for commercial viability

# Competitive exclusion



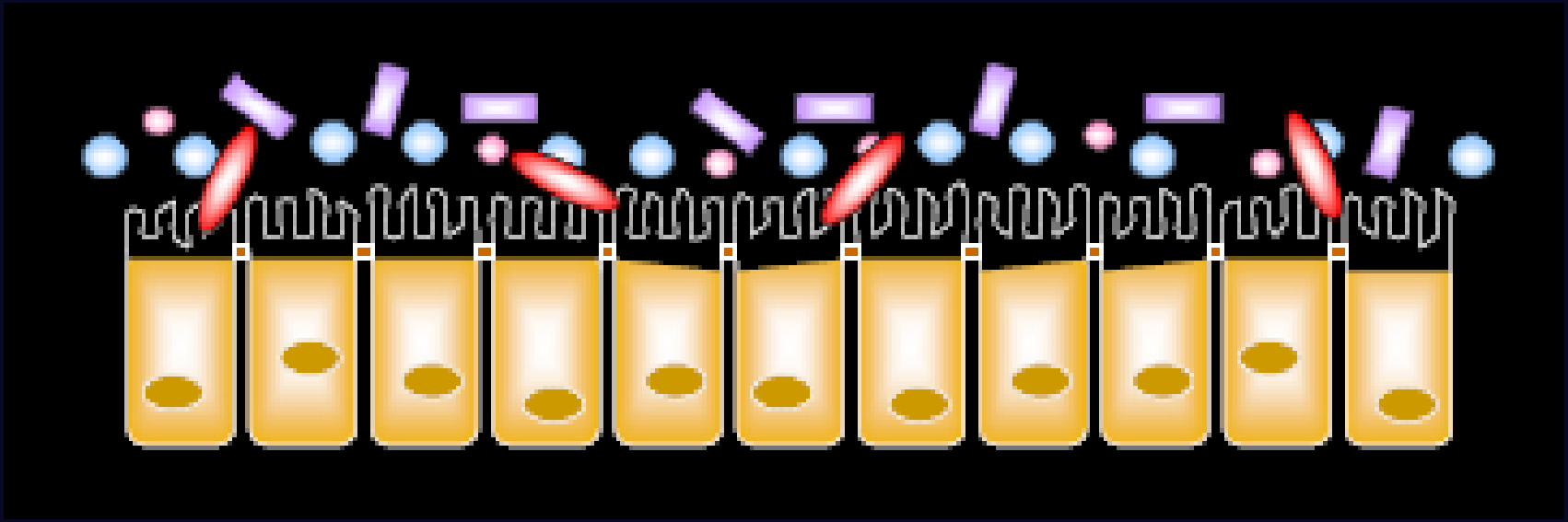

http://wab.medvet.umontreal.ca/wab/sitee/prog/ewaschuk.pdf

# Production of antibiotics




http://wab.medvet.umontreal.ca/wab/sitee/prog/ewaschuk.pdf

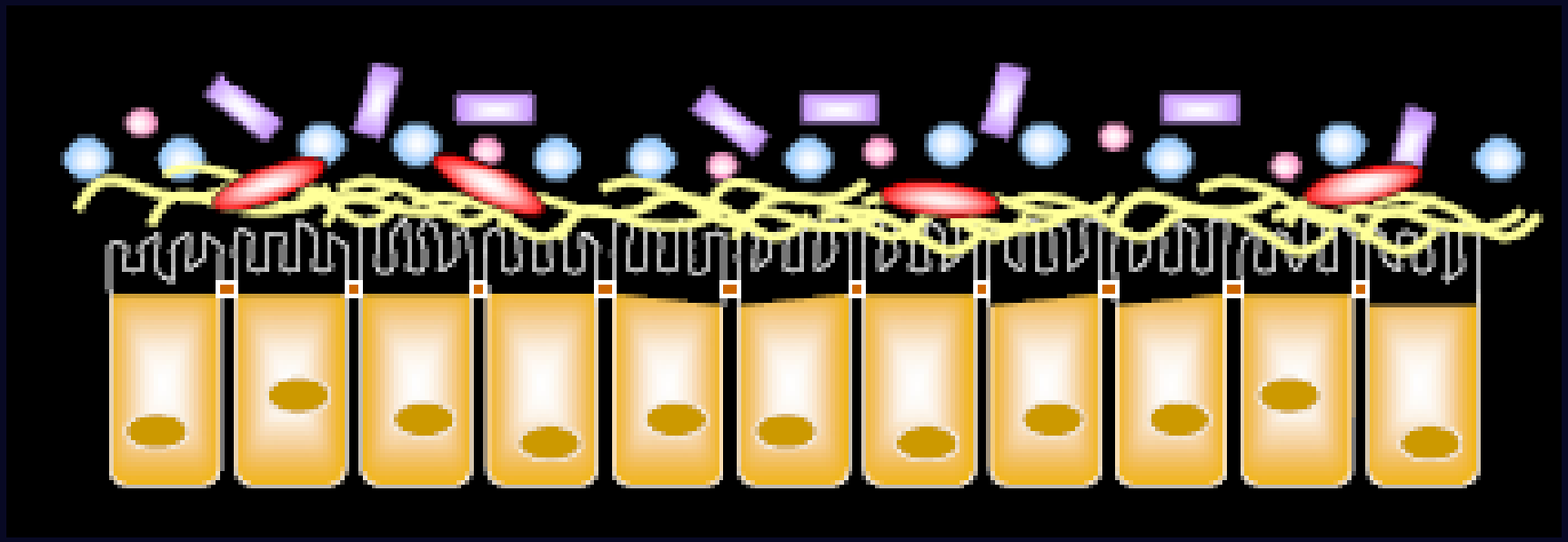
# Improve barrier function



http://wab.medvet.umontreal.ca/wab/sitee/prog/ewaschuk.pdf

# Increased mucus secretion

- Upregulation of mucin producing genes (MUC2, MUC3)
- Mucus preservation: probiotics unable to degrade



http://wab.medvet.umontreal.ca/wab/sitee/prog/ewaschuk.pdf

# Increase immune function

- Increased production of IgA, IgE, IgG
- Cytokines:IL-1 $\beta$ , IL-8, IL-10, IL-4, TNF- $\alpha$

http://wab.medvet.umontreal.ca/wab/sitee/prog/ewaschuk.pdf

# 乳酸菌之篩選及菌種鑑定方法

動物糞便

泡菜


酸酵乳

待測樣品經連續稀釋，使用倒皿法製作 medium

最適溫度培養形成菌落 → 檢測每g(ml)所含菌量

挑選菌落

菌株保存及生長型態確認純種分離株 → 純種分離株

進行菌種鑑定
①Gram
②鏡檢法
③Endospore
④Catalase測試
⑤糖類醱酵試驗
⑥產氣試驗
⑦生長溫度試驗
⑧耐鹽性試驗

菌種鑑定由API system test 確認

# 益生菌篩選流程

乳酸菌之篩選及鑑定

↓

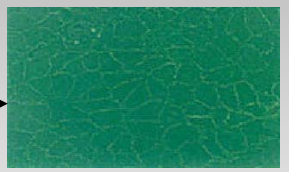
耐膽鹽性分析

↓

吸附性試驗 ← 耐酸性分析 → 抑制腸道菌試驗

↓

醱酵寡糖分析



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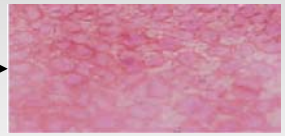
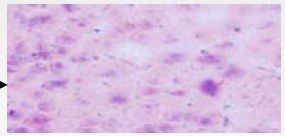
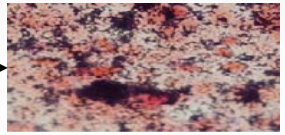


Negative control



Positive control



Isolated strain

菌株吸附至腸道細胞

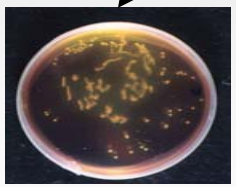
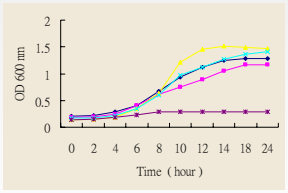
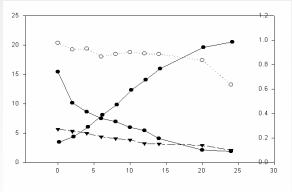


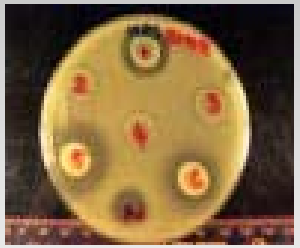
Plate 檢測




生長密度



HPLC 分析



抑制腸道害菌生長

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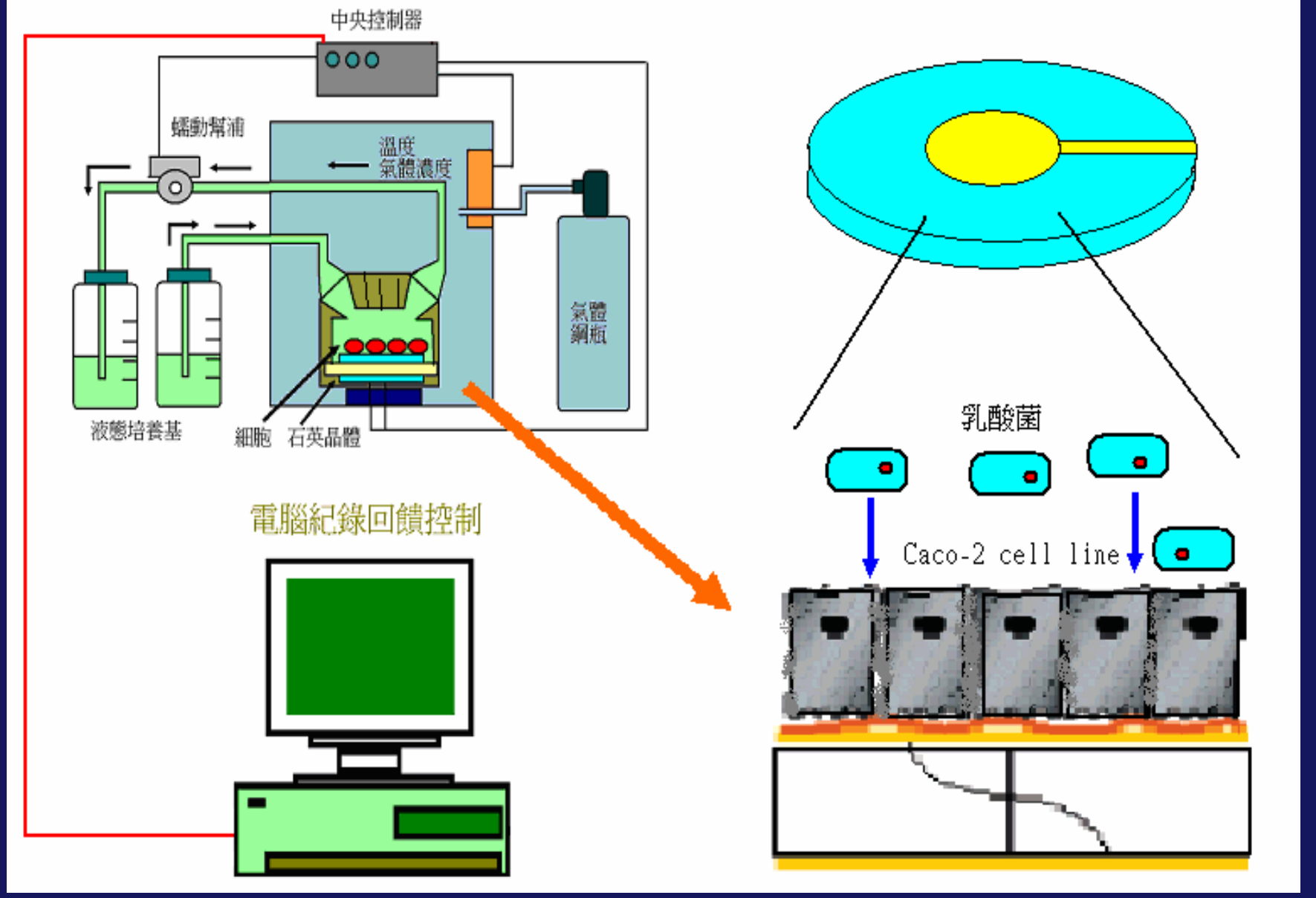
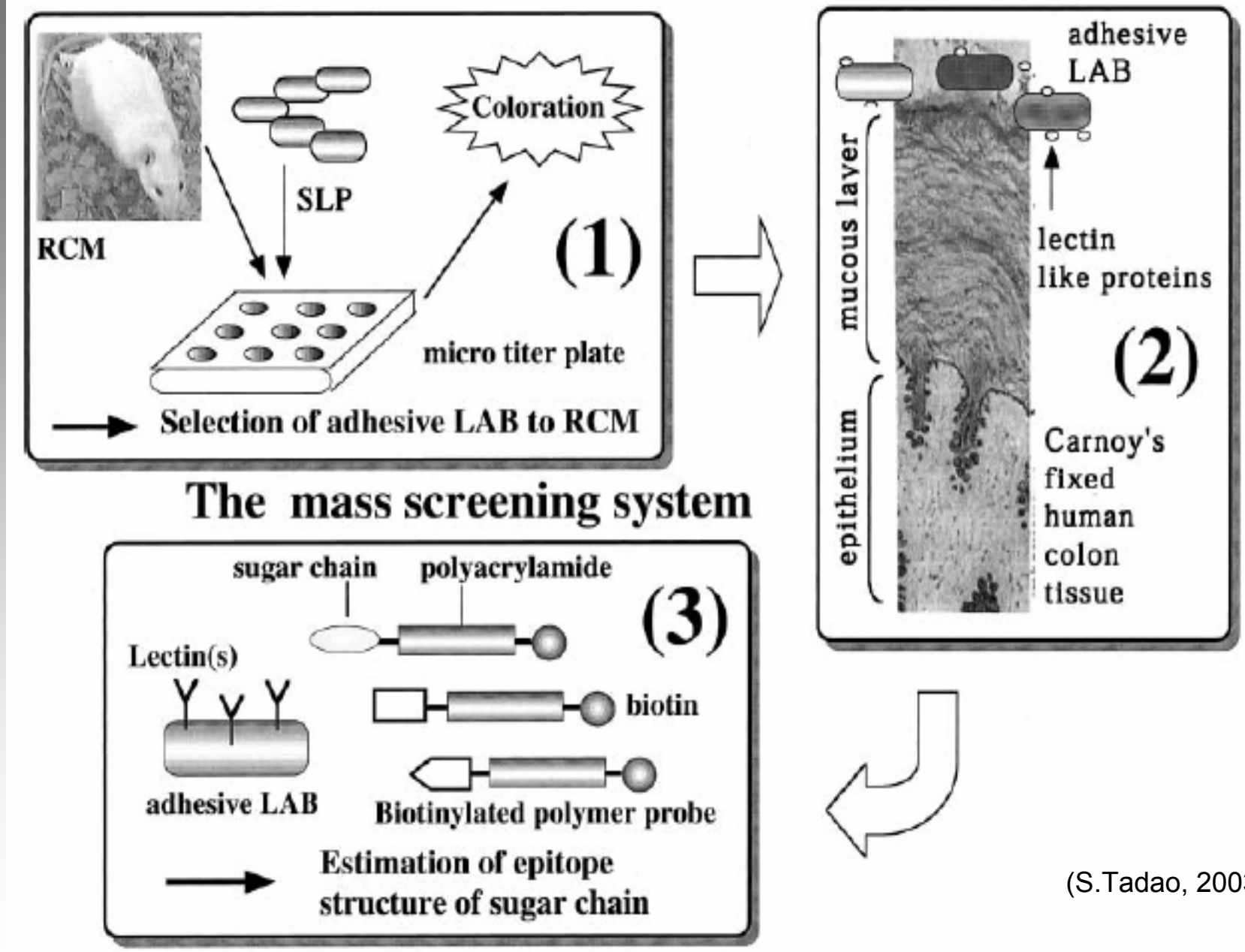

中央控制器
蠕動幫浦
溫度
氣體濃度
氣體鋼瓶
液態培養基
細胞
石英晶體
電腦紀錄回饋控制
乳酸菌
Caco-2 cell line




(S.Tadao, 2003)

Fig. 2 A proposed mass screening system for probiotic LAB with high adhesion to human colon [illegible]a. RCM, rat colonic mucin; SLP, surface layer protein; LAB, lactic acid bacteria.

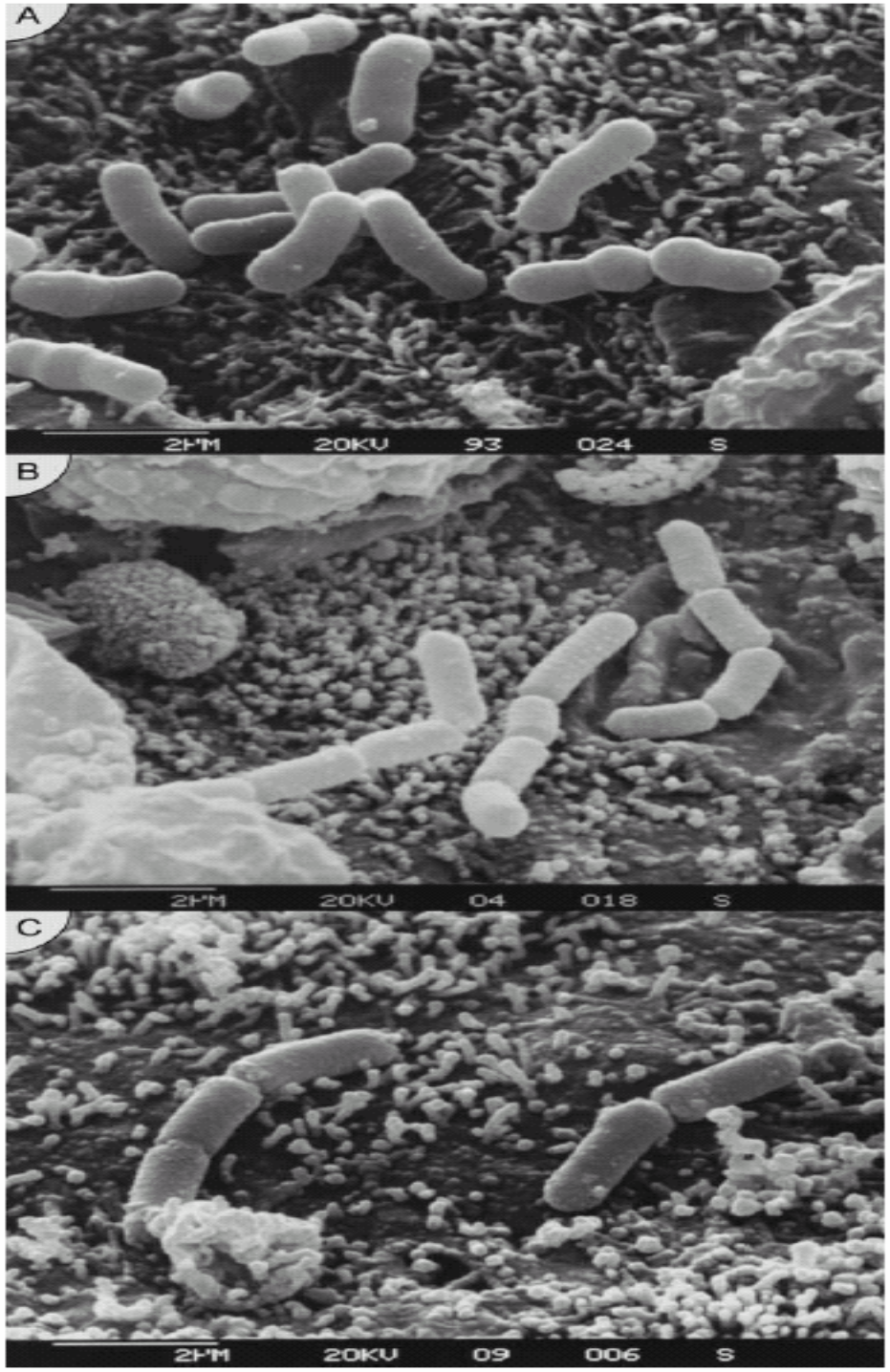



Fig. 1. **Examination by electron microscopy of adherence of probiotic strains to differentiated human intestinal epithelial cells.** (A). A Monolayer of HT-29 covered with *B. lactis* DR10. (B) Adhesion of *L. rhamnosus* DR20 on monolayer of Caco-2 cell monolayer. (C) Adhesion of *L. acidophilus* HN017 to HT-29 cell monolayer. In all three photographs, the probiotic strains are seen adhering to microvilli that form the brush border of HT-29 or Caco-2 cell monolayers magnification (10,000x). (Gopal P. K. et al., Inter. J. Food Micrubiol**.** 67:207-216., 2001)

**Table 4**. Comparison of adherence properties of NCFM and other probiotic lactobacilli. +, adherence observed; –, no adherence; HT29, indicates HT29 or HT29-MTX cells.

| *Lactobacillus* species and strain | Cell line used in adherence assay: HITH s0074 | Caco-2 | HT29 | Factors involved in adherence | Reference |
|---|---|---|---|---|---|
| *L. acidophilus* NCFM | + | + | + | Protein | Greene and Klaenhammer, 1994 |
| *L. acidophilus* LA | + | + | + | Protein | Chauviere et al., 1992; Coconnier et al., 1992 |
| *L. acidophilus* LB | | + | + | Secreted protein | |
| *L. acidophilus* LA1 | | + | + | Secreted protein | |
| *L. crispatus* BG2FO4 | + | + | + | Protein and carbohydrate | Greene and Klaenhammer, 1994 |
| *L. gasseri* ADH | + | + | + | Carbohydrate | |
| *L. rhamnosus* GG | | + | + | | Mack et al., 1999 |
| *L. plantarum* 299 & 299V | | | + | Mannose-adhesion | Adlerberth et al., 1996 |
| *L. delbrueckii* 1489 | + | + | | | Greene and Klaenhammer, 1994 |

NCFM is the progenitor of the strain being used for complete chromosome sequencing and therefore will be a cornerstone
strain for understanding the relationship between genetics and probiotic functionality. (M. E. Sanders et al., 2001)

Table 1. Adhesion of three lactobacilli strains, *L. rhamnosus* GG, *L. gasseri* K7 and *L. gasseri* LF221 to Caco-2 cell line; the values (cfu/Caco-2) for two concentration levels of lactobacilli were derived from the linear regression analysis

| Test strain | pH | Adhered bacteria (cfu/Caco-2) | |
|---|---|---|---|
| | | $2.5 \cdot 10^6$ cfu/well (=5.2 cfu/Caco-2) of added bacteria | $2.5 \cdot 10^8$ cfu/well (=520 cfu/Caco-2) of added bacteria |
| K7 | 7 | 0.006 | 3.25 |
| | 4.5 | 0.014 | 6.44 |
| LF221 | 7 | 0.1 | 1.64 |
| | 4.5 | 0.41 | 10.44 |
| GG | 7 | 0.009 | 3.7 |
| | 4.5 | 0.018 | 16.93 |

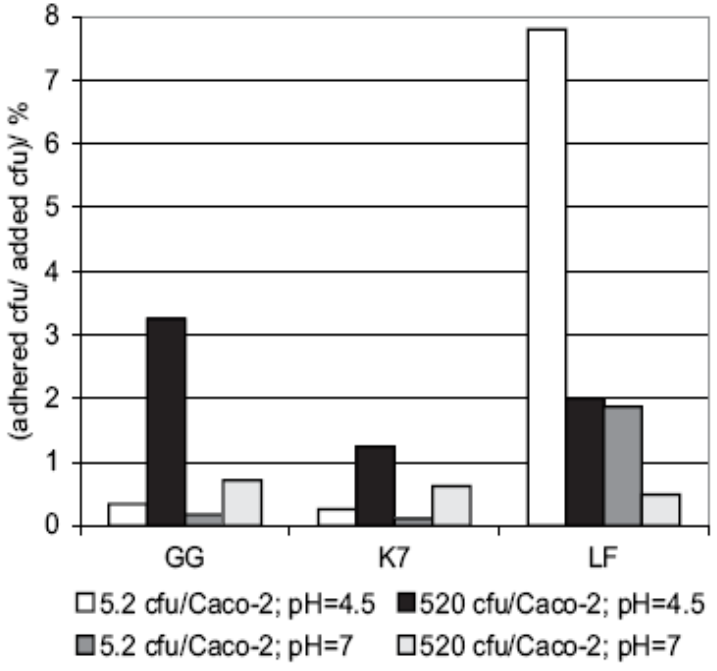



**Fig. 3. Efficiency of adhesion expressed as the ratio (%) of lactobacilli viable cells that remained adhered to the Caco-2 enterocytes.** The ratio was calculated from the values obtained by linear regression analysis

(B.M., Bojana et al., 2003)

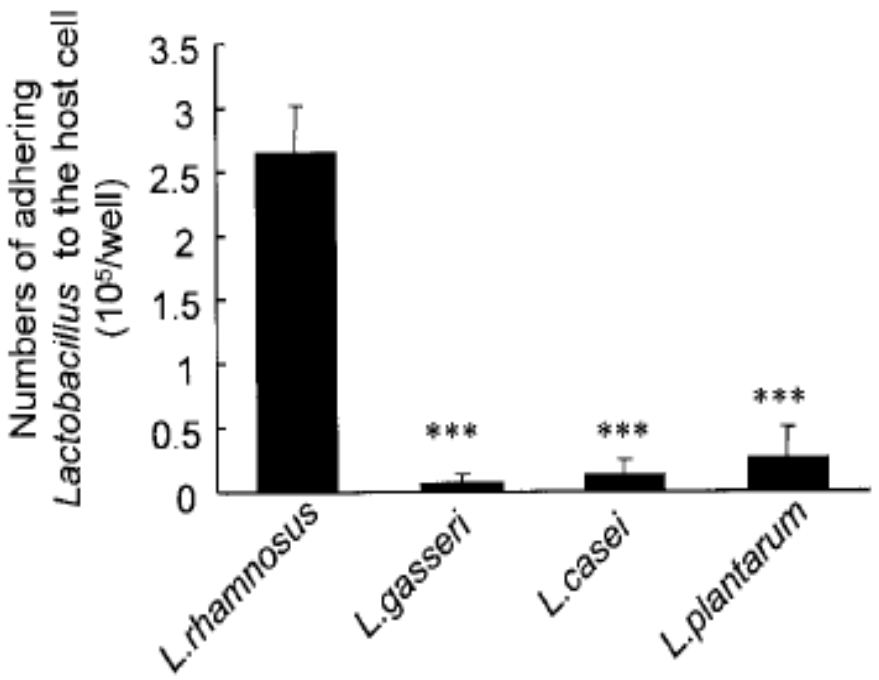
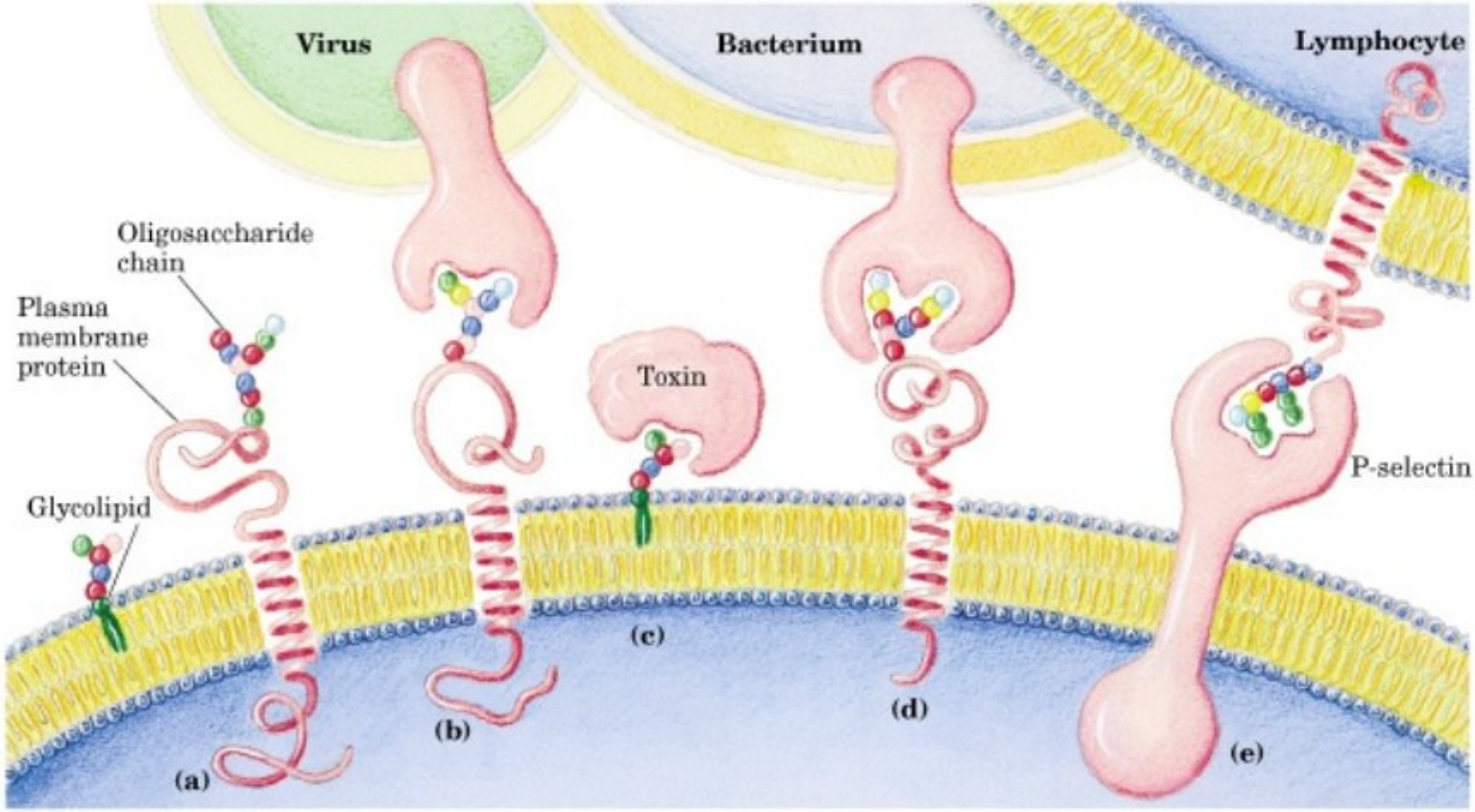



Fig. 5. Adhesion of *Lactobacillus* strains to a human epithelial cell line. *Lactobacillus* strains were added to C2BBe1 and incubated for 3 hr. After being washed thoroughly, cells were lysed and aliquots were plated on MRS agar as described in "Materials and Methods." ***$P$-value<0.001.

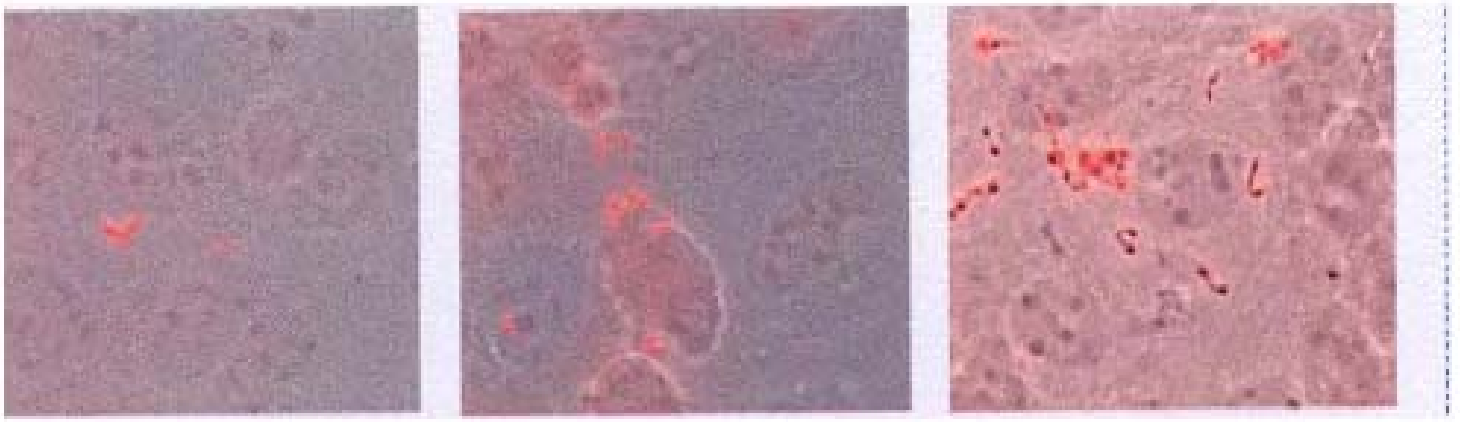
(H. Jyunko et al., 2003)

# Saccharides and Cell Adhesion




http://courses.cm.utexas.edu/archive/Spring2002/CH339K/Robertus/overheads-2/ch9_cell-adhesion.jpg

# In vitro adhesion



LOW → HIGH

Caco2 cells monitored with FISH analysis with different level of adhesion

# 乳酸菌的腸道吸附實驗

Seeding the Caco-2 cell line on wells

↓ 14-20 days

After Caco-2forming confluent monolayer with apical microvilli and several 'brush-border‘ hydrolases (Pinto et al., 1983), adding fixed Lactobacillus on the layer.

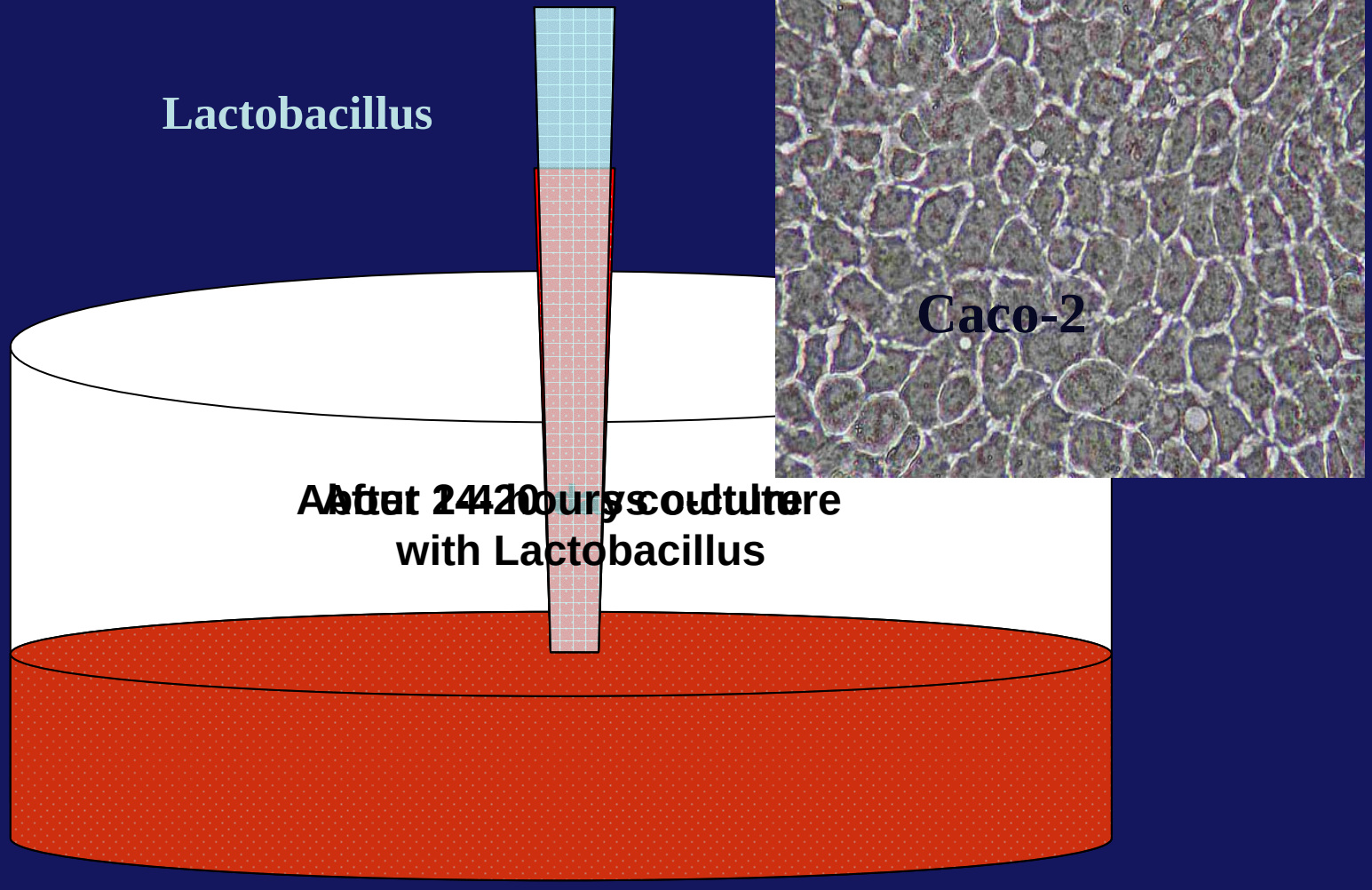
↓ 2-3 hours

Discard the culture medium → Gram stain

↓

Observe stained cells under phase-contrast light microscopy

# Adhesion assay of Caco-2 cell line

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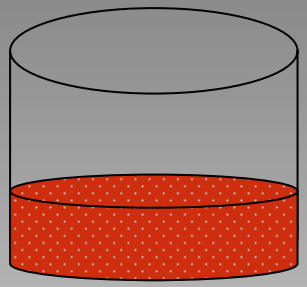
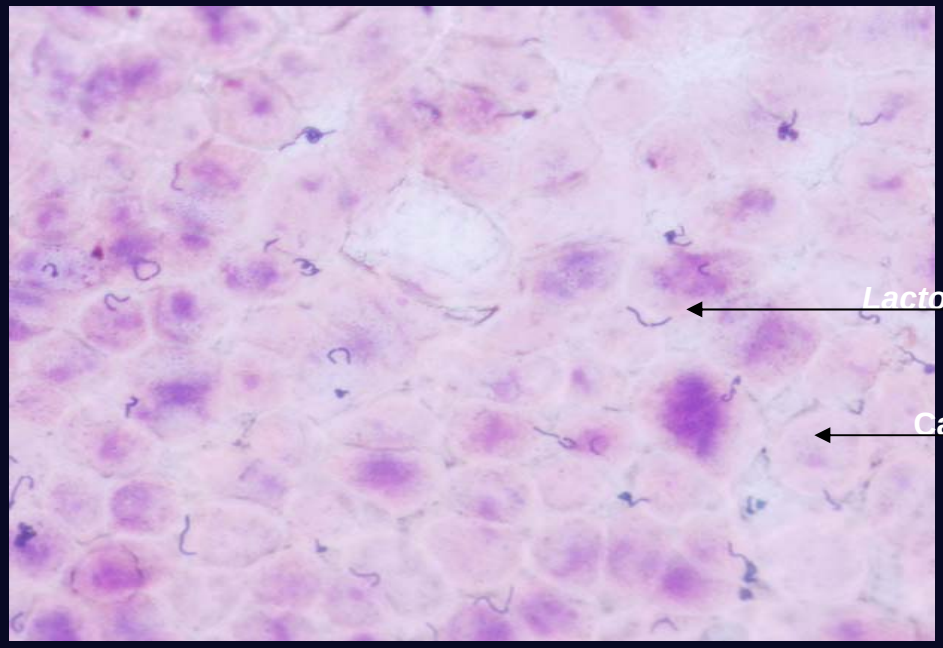
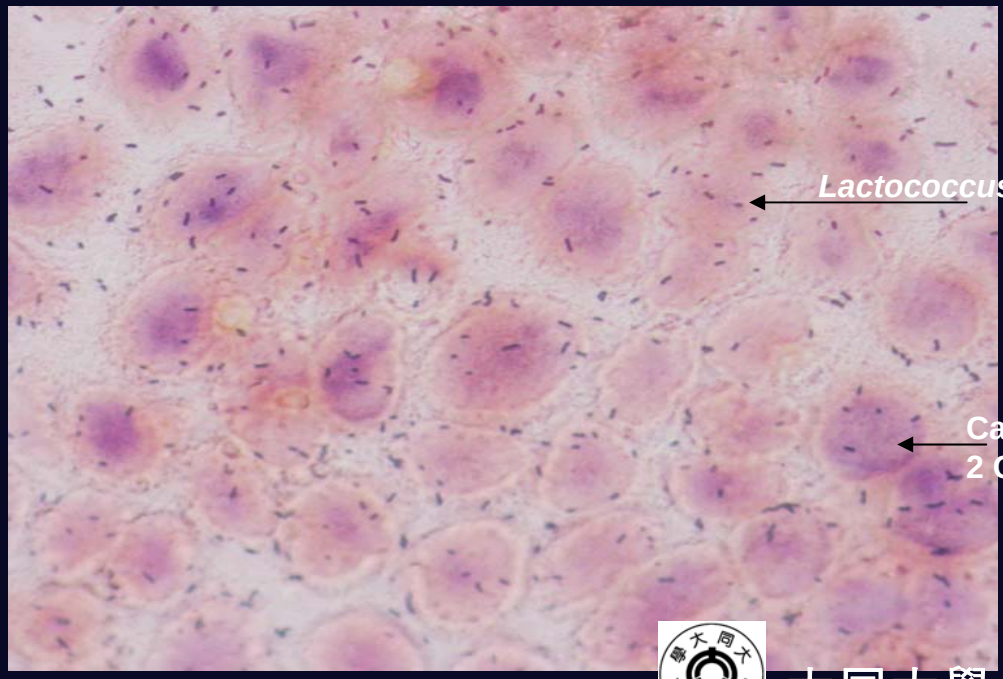
# Adhesion assay of Caco-2 cell line



## Gram stain

1. air dry (prepare a smear)
2. Primary stain (Crystal Violet stain for 30sec to1min)
3. Rinse with water
4. Fixing agent (Iodine solution fix for 30sec to 1min)
5. Rinse
6. Decolourize (wash with 95% alcohol for 5sec)
7. Rinse with water
8. Counter stain (Safranin stand for 30sec)
9. Rinse with water
10. Blot dry
11. Observe under  Oil Immersion




(Chi-Lin Tsai, 1992)

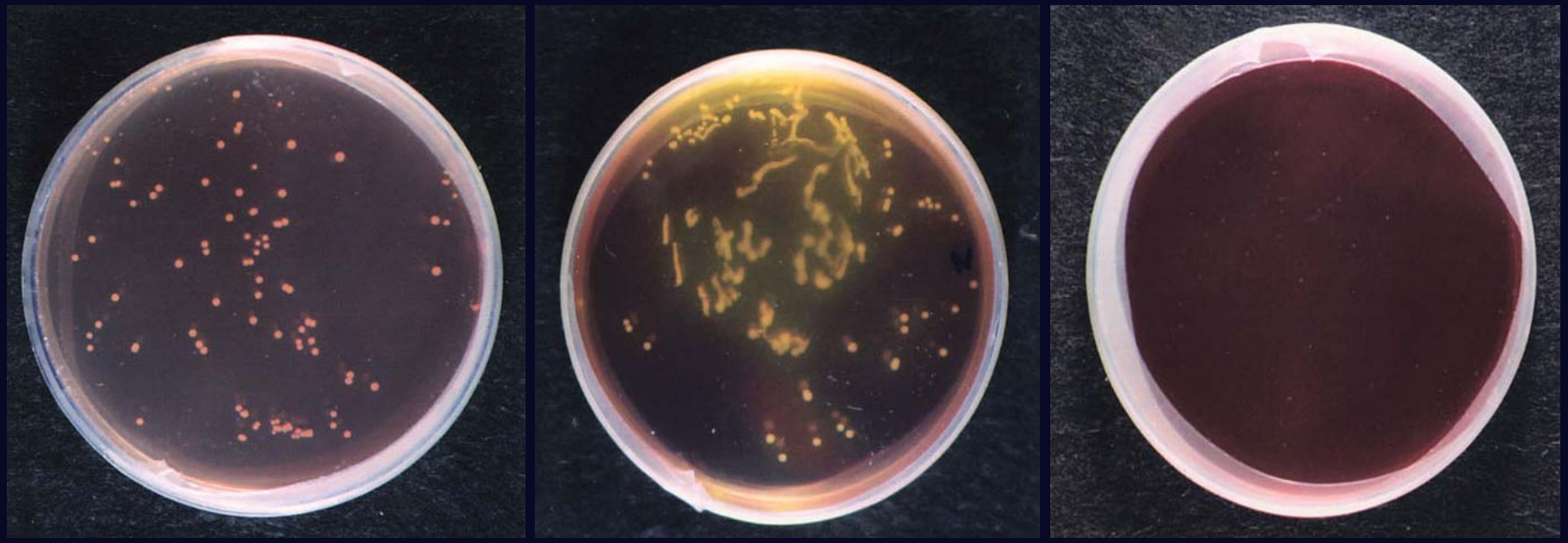
# 乳酸菌分離株在體外小腸模擬系統的吸附情形

| Strain | LAB adherence count (CFU /100 cell)** | Determine to adherence*** |
|---|---|---|
| LGG* | 45 | + |
| E55 | 10 | − |
| E47 | 3 | − |
| V12 | 245 | ++ |
| E53 | 12 | − |
| E30 | 8 | − |
| E51 | 238 | ++ |
| E38 | 78 | + |
| E15 | 38 | + |
| V52 | 4 | − |

•33株分離株中，有14株分離菌株可以吸附至Caco-2細胞株上，其中又以菌株V12、E51可以強烈吸附至Caco-2 細胞株上

(Chi-Lin Tsai, 1992)

（A）　　（B）　　（C）



圖八、以含有寡糖之MRS plate進行醱酵寡糖特性分析模式圖。（A）乳酸菌不醱酵prebiotics物質之負對照組，其在菌落周圍不產生黃色區帶，（B）乳酸菌可醱酵prebiotics物質之正對組，其在菌落周圍產生黃色區帶，（C）不添加乳酸菌之prebiotics-MRS plate背景顏色。

# 表、乳酸菌分離株之醱酵一些寡糖之分析

| Strain No. | Oligosaccharides | | |
|---|---|---|---|
| | FOS | IMOS | GOS |
| E55 | ＋* | ＋ | ＋ |
| E47 | － | ＋ | ＋ |
| V12 | － | ＋ | ＋ |
| E53 | － | ＋ | ＋ |
| E30 | ＋ | ＋ | ＋ |
| E51 | － | ＋ | ＋ |
| E38 | － | ＋ | ＋ |

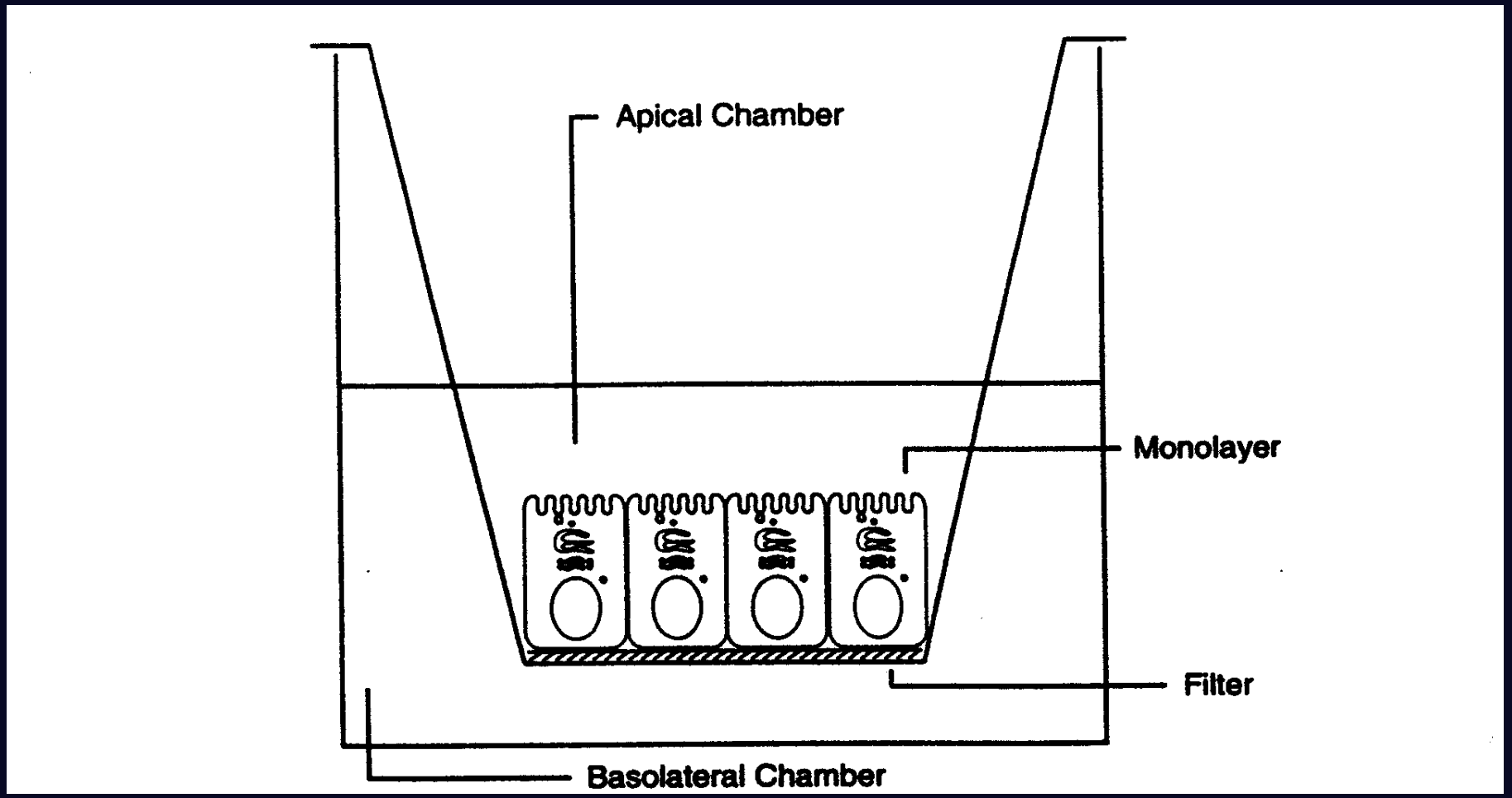
- 33株乳酸菌分離株在醱酵果寡糖能力上較低，只有9個乳酸菌菌落可醱酵果寡糖，另24個乳酸菌無法醱酵果寡糖。在醱酵異麥芽寡糖及龍膽寡糖能力上較佳，33株乳酸菌分離株均能醱酵。

# Probiotics properties of the isolated lactic acid bacteria

| Isolated colony | *Acid treatment | ** Bile salt treatment | Adsorption *** | Prebiotics digestion*** |
|---|---|---|---|---|
| | pH 3.0 | 0.3% 之 OD 600 nm | | |
| *Lactobacillus* sp. B2 | 7.3 × 104 | 2.059 | ＋＋ | ＋ |
| *Lactobacillus* sp. B6 | 2.2 × 104 | 2.120 | ＋＋ | ＋ |
| *Lactobacillus* sp. B7 | 4.3 × 104 | 2.093 | ＋＋ | ＋ |
| *Lactobacillus* sp. B8 | 3.3 × 104 | 2.154 | ＋＋ | ＋ |
| *Lactobacillus* sp. B12 | 7.8 × 104 | 2.201 | ＋＋ | ＋ |
| *Lactobacillus* sp. B14 | 5.5 × 105 | 2.209 | ＋＋ | ＋ |
| *Lactobacillus* sp. B14 | 1.1 × 104 | 2.109 | ＋＋ | ＋ |
| *Lactobacillus* sp. D15 | 3.5 × 105 | 2.307 | ＋＋ | ＋ |

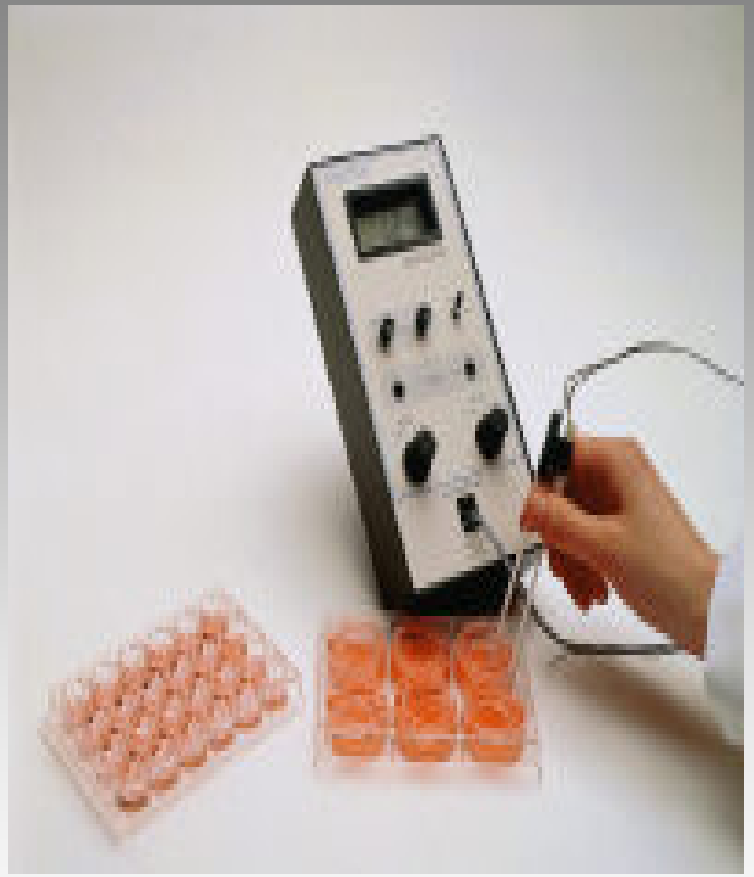
# Transwell® polycarbonate filters..

Hidalgo 1996 in Borchardt, Smith and Wilson (eds), Models for Assessing Drug Absorption and Metabolism.New York, Plenum Press: 35 - 50.



# Transepithelial Permeability Experiments

47-79代的Caco-2細胞於輸送實驗前20-35天培養在Transwell中，通過細胞單層膜的TEER值（transepithelial electrical resistance）以Millicell-ERS Voltohmmeter來測量，並選用在細胞培養基中TEER值＞350Ω×cm²的inserts進行實驗。inserts以溫的transport medium（具有25mM HEPES且pH值爲7.4的Hank's Balance Salt Solution）沖洗30分鐘。（Richard A. Walgren, 1998，Biochemical Pharmacology 55, pp.1721-1727)



Millicell-ERS Voltohmmeter---millipore

# 計算物質通透量以Papp值表示:

$$P_{app} = \left( \frac{V}{A \cdot C_o} \right) \times \frac{dC}{dt}$$

A：單層膜表面積
V：接受部位的容積
$C_0$：加入部位Sample的初始濃度
dC/dt：通透速率

# Gentamicin

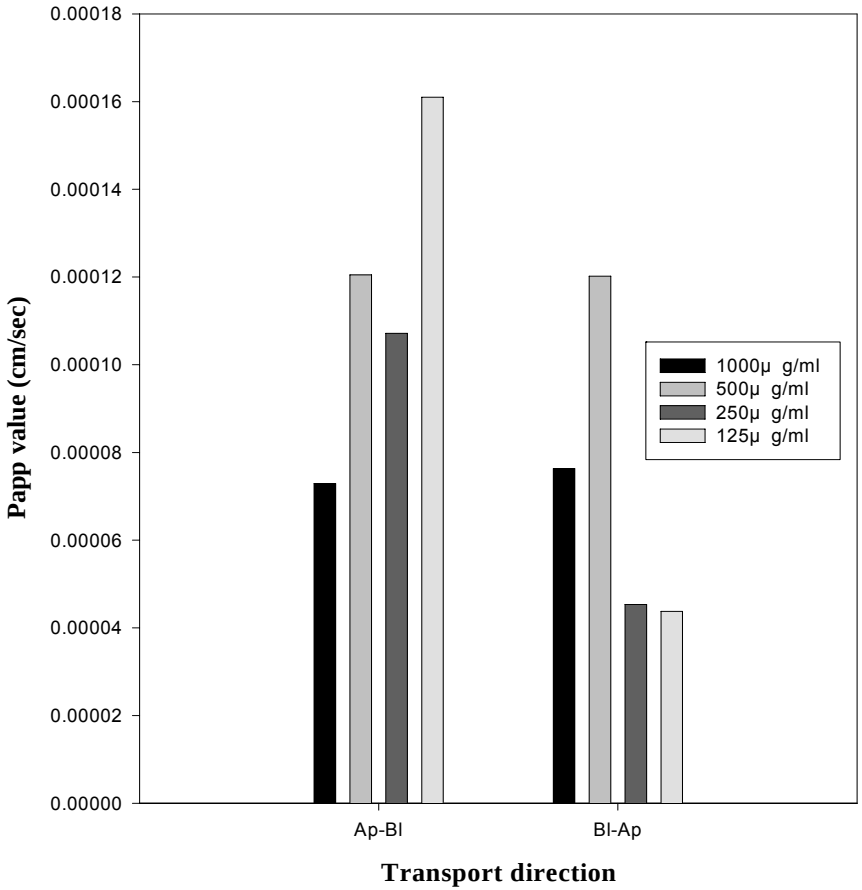



Fig. 21. The result of Gentamicin transport through Caco-2 monolayer.

對不同濃度的Gentamicin在Caco-2細胞單層膜上通透的結果顯示(見Fig.21)，在濃度為1000及500 μg/ml時，Gentamicin雖然都可以通透，但是Ap-Bl的通透係數與Bl-Ap十分接近，此一現象可以用臨床上Gentamicin因吸收效果不好故皆以針劑給藥而非口服來解釋 。

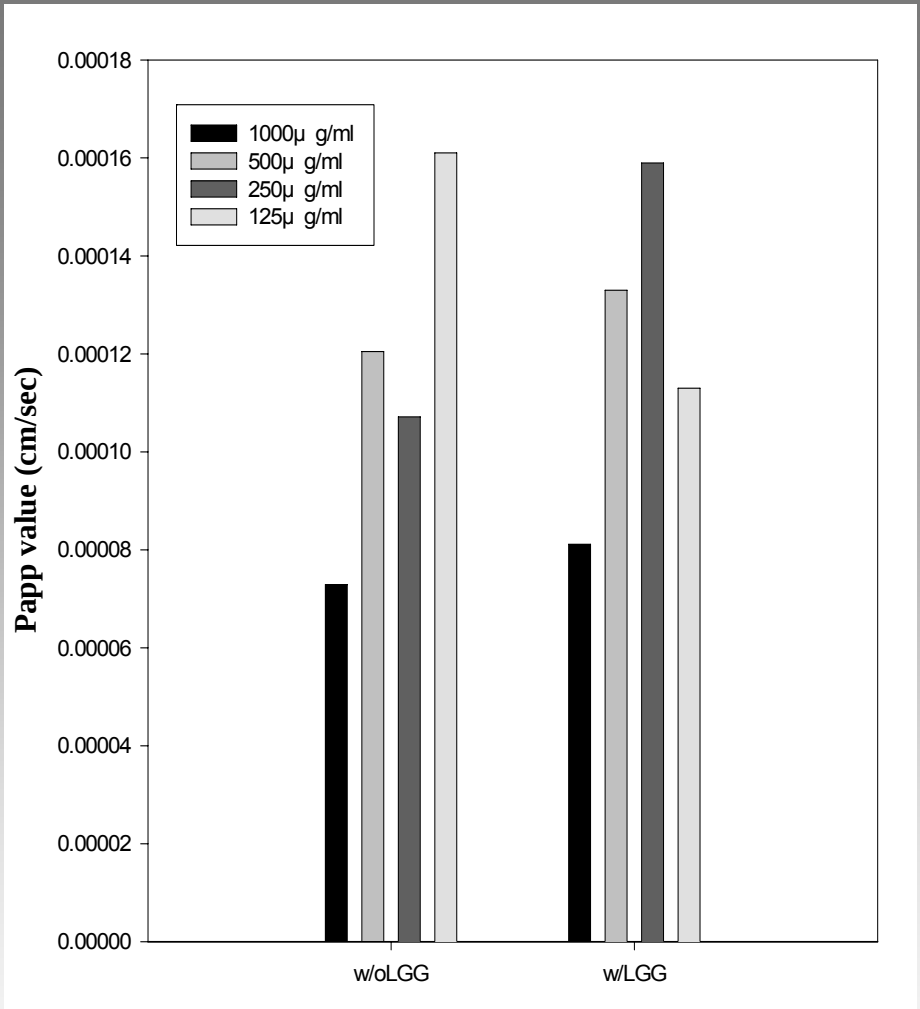



Fig. 22. Comparison of the effect of *L.* GG adhesion to Gentamicin transport. (w/o:without; w/:with)

經過乳酸菌吸附的前處理之後，Gentamicin濃度較高的前三組的通透皆有些許增加，但在低濃度125 μg/ml則無此一現象(見Fig.22)。

# *L.GG*抑制*C. difficile*生長實驗

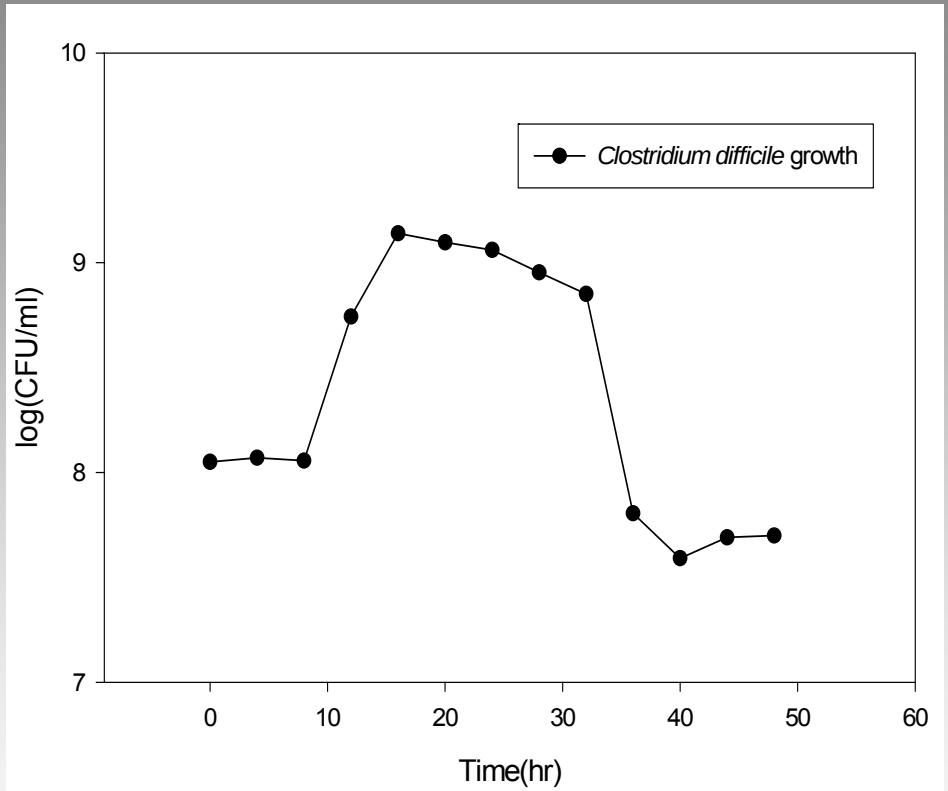



Fig.7.L.GG抑制*C. difficile*生長，第16小時*C. difficile*生長變慢，至第36小時生菌數下降。

# Caco-2細胞單層膜染色

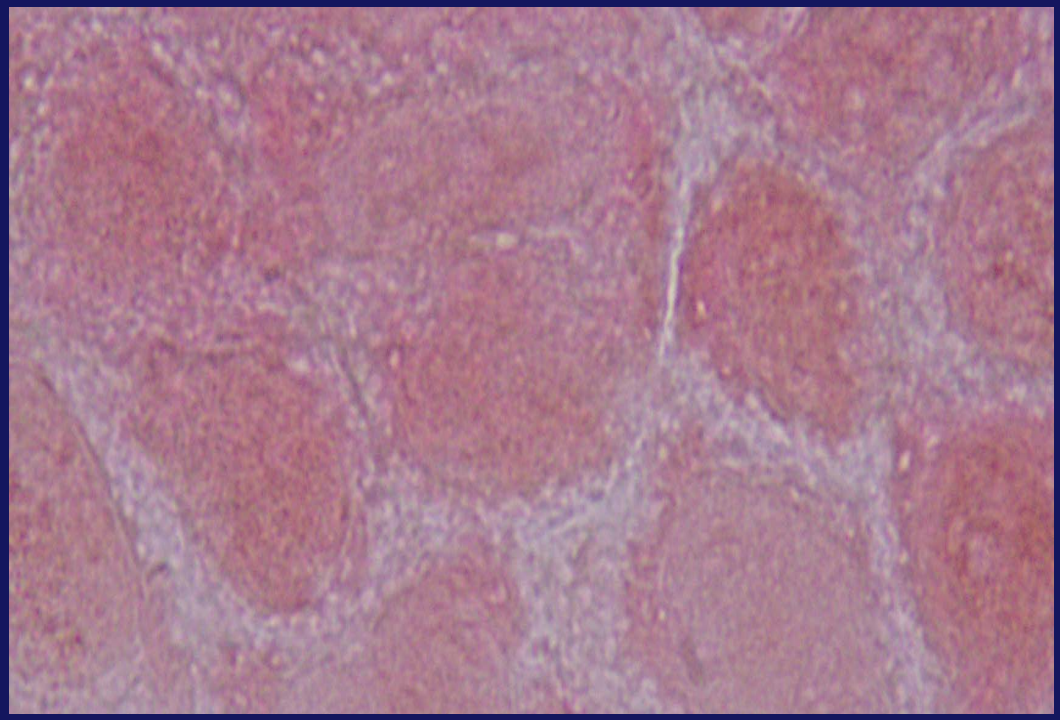


Fig. The Gram staining result of C.Caco-2 monolayer (magnification 1000×).

# Caco-2細胞單層膜+ *C. difficile*

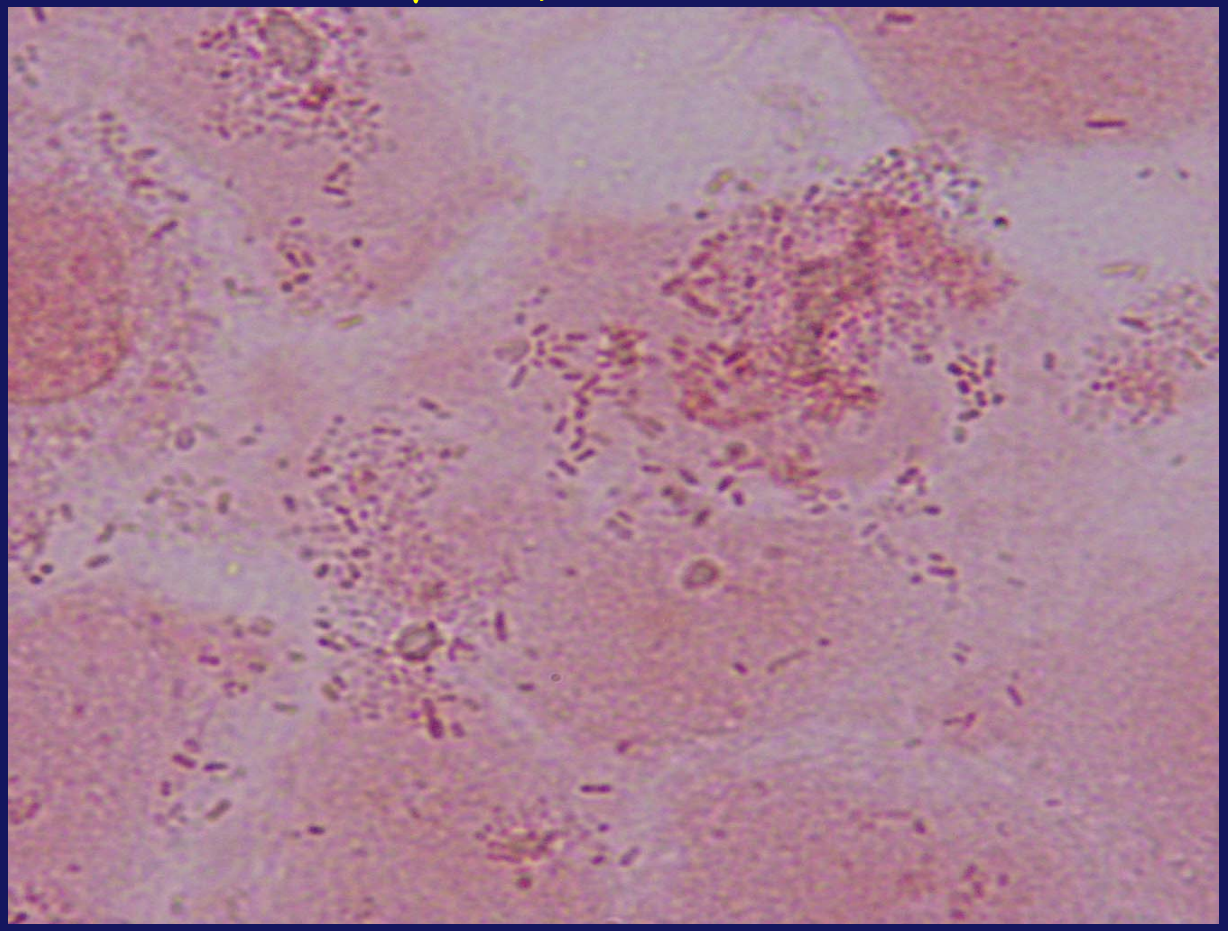
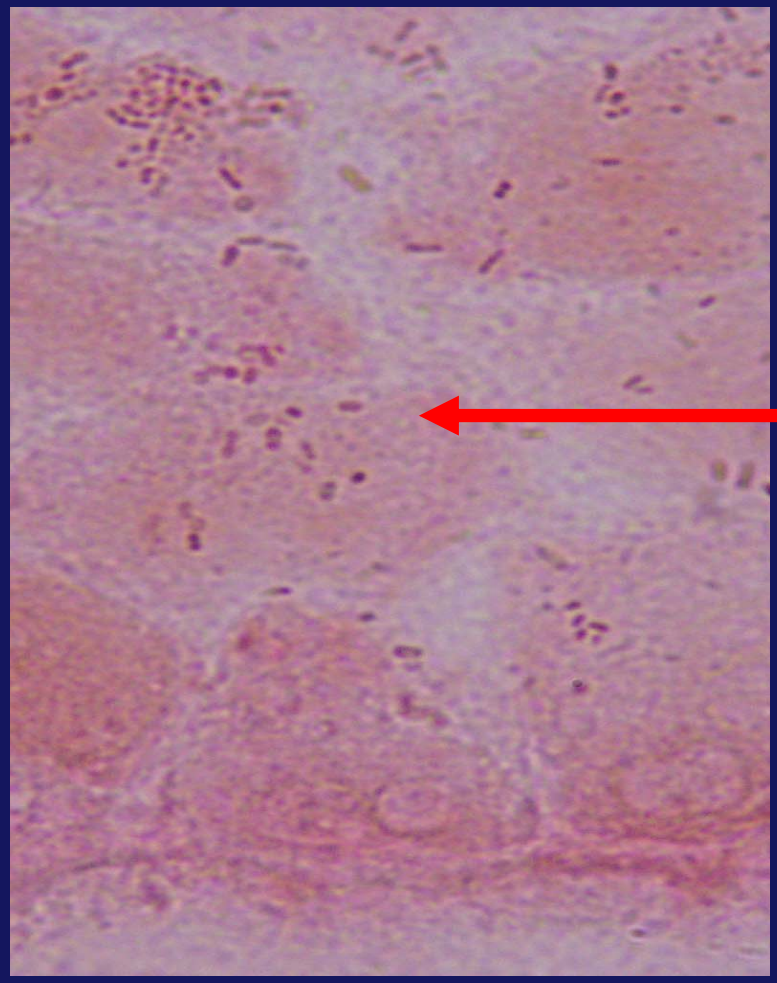


**Fig. The Gram staining result of *C. difficile* adhered to Caco-2 monolayer (2hr) (magnification 1000×).**

# *C. difficile*破壞Caco-2細胞單層膜



*C. difficile*可觀察到大多數的細菌會黏附在細胞接合處

Fig. 10. The Gram staining result of *C. difficile* adhered to Caco-2 monolayer (48 h) (magnification 1000×).

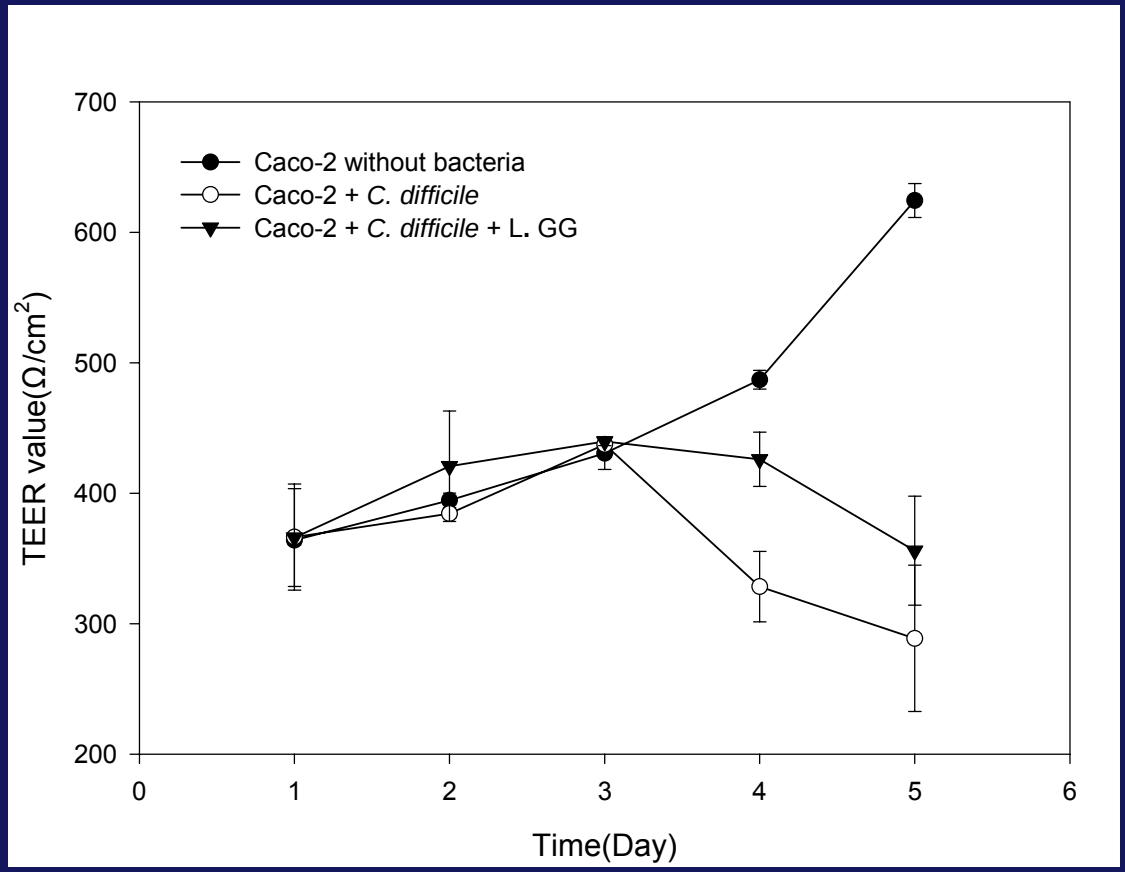


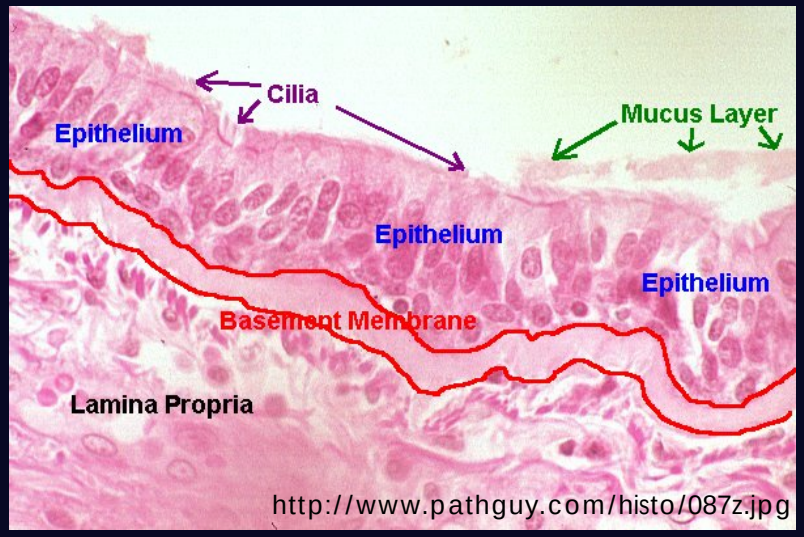

Fig. Effect of co-culture of ***L.GG*** on disruption of Caco-2 monolayer by ***C. difficile***.

# Immuno-mechanism of probiotic LAB which adhered to Caco-2 cell

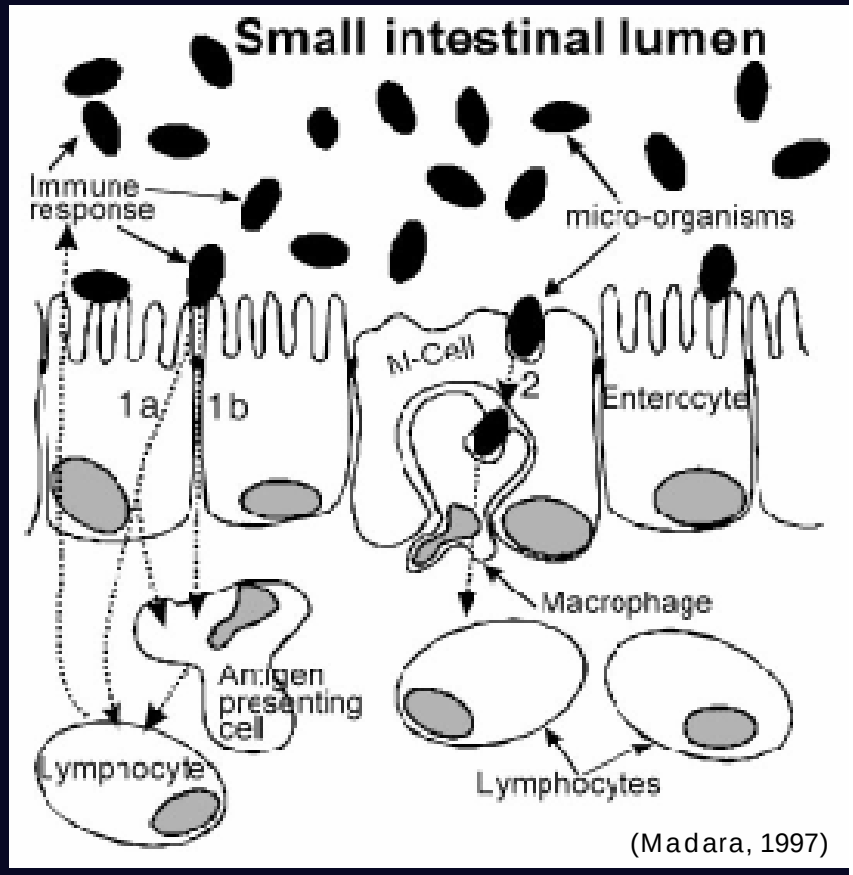
大同大學 生物工程系

# • 免疫調節與益生菌黏附的關係：

◆ 為影響免疫系統，益生菌必須活化腸道淋巴組織的淋巴細胞，它會擴散分布於上皮細胞間，殖生於lamina propria與sub-mucosa的位置。




較小的抗原攝食會直接穿越正常的上皮細胞發生(路徑1a)；或透過細胞間接合處漏洞(路徑1b)發生，藉由益生菌所在位置使免疫反應發生而產生代謝物，有利於免疫的發展，而非純粹耐受性的強化。




Possible ways of stimulation of the immune system by ingested probiotics.

(S.Tadao, 2003)

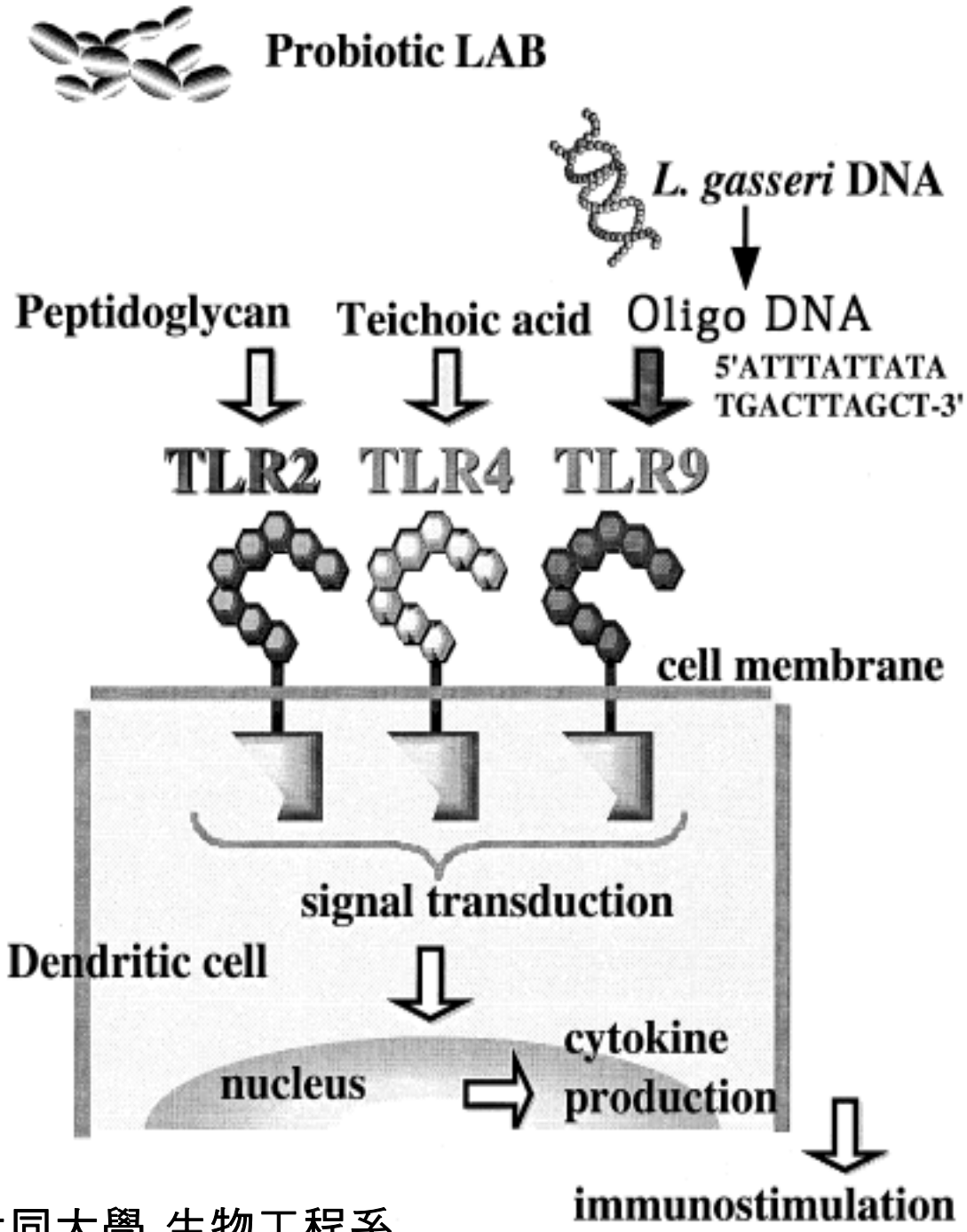
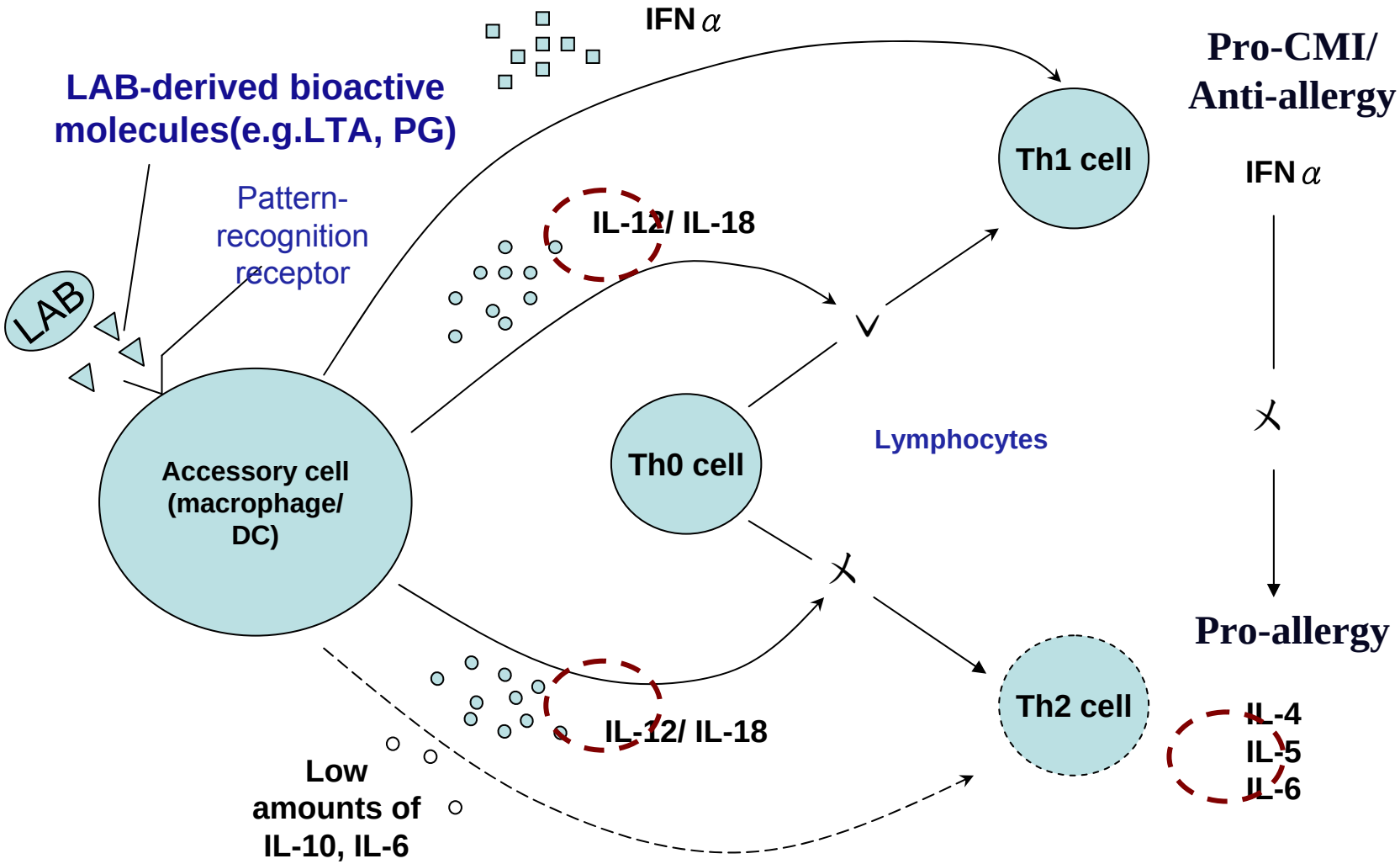
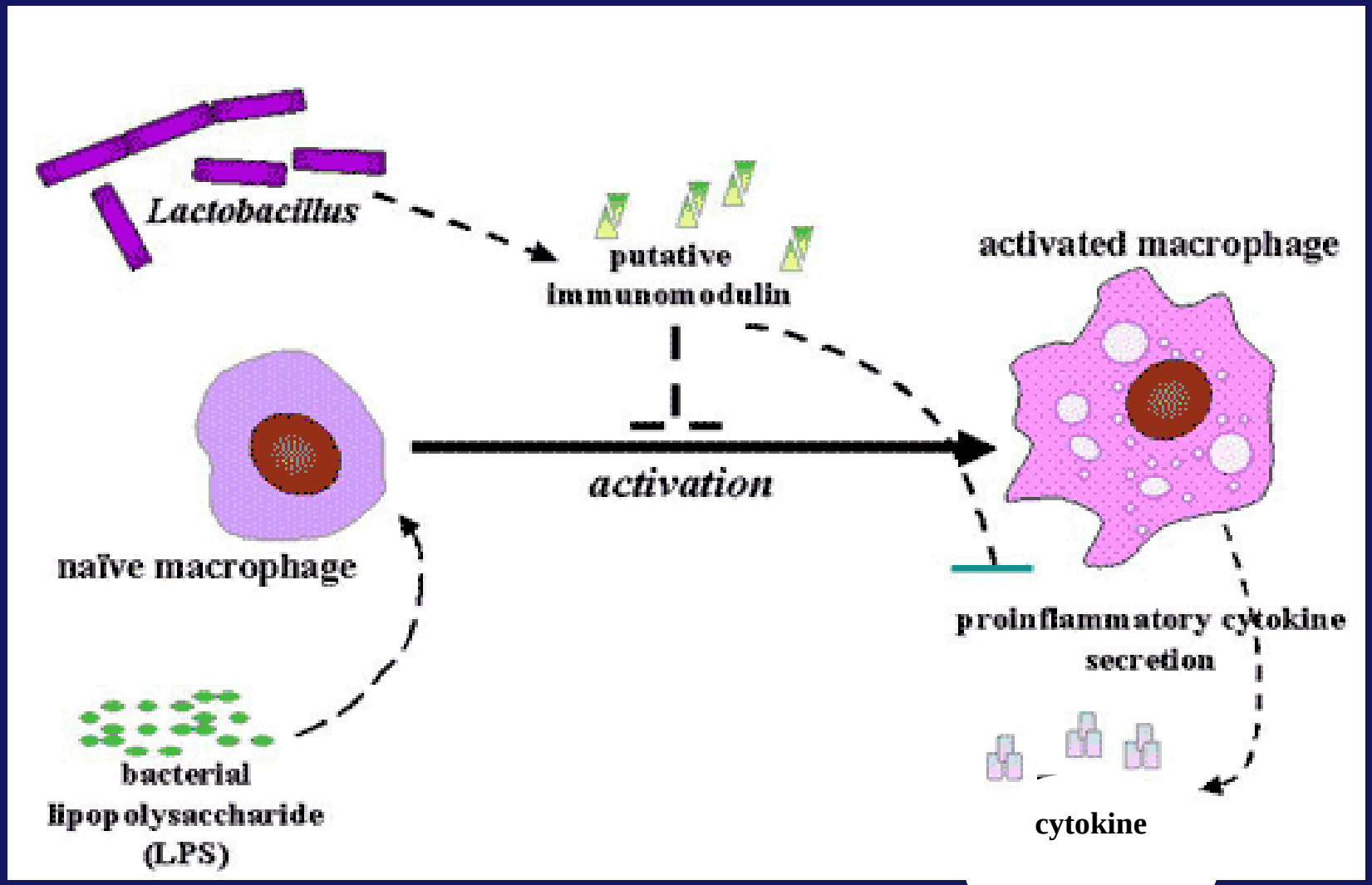



Fig. 4 Estimated pathway of immunostimulation reaction by peptidoglycan, teichoic acid and oligo DNA motif from *Lactobacilus gasseri*. LAB, lactic acid bacteria; TLR, toll-like receptor.




Theoretical model for anti-aalergy mechanisma of immunoregulating lactic acid bacteria(LAB). NB：Th cell TCR interaction with MHC II-bound allergenic peptide has been omitted for clarity.

(M.L. Cross et al., 2001)


Lactobacillus
putative
immunomodulin
activated macrophage
activation
naïve macrophage
proinflammatory cytokine
secretion
bacterial
lipopolysaccharide
(LPS)
cytokine

# 免疫檢測技術(動物免疫細胞內的cytokine含量)

RAW264.7

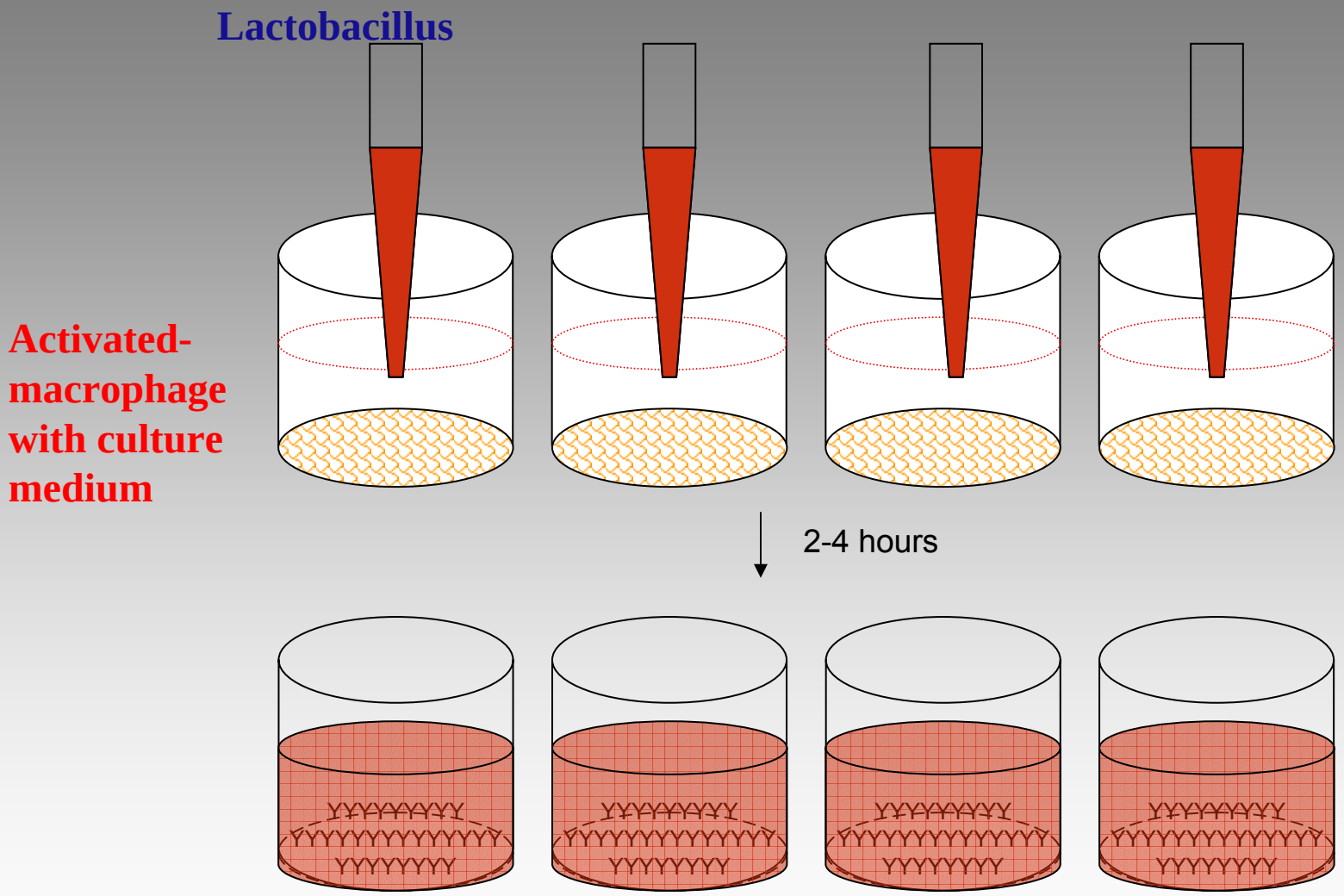
J774A.1

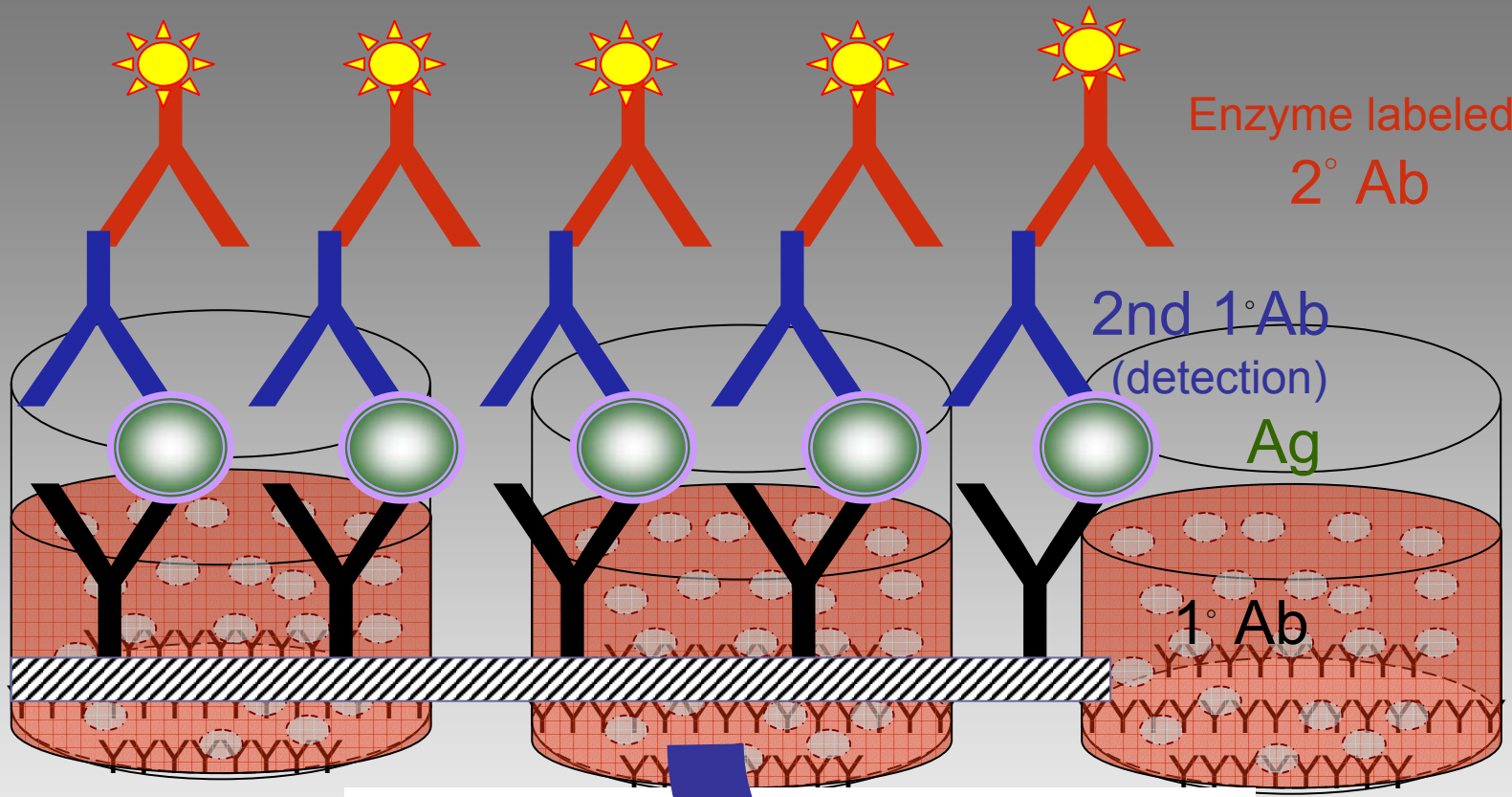
Stable expression of either mutant in RAW264.7 (which are more amenable to transfection than. J774A.1)

Macrophage stimulated by Lactobacillus (2-4 hours)

Exam expression of cytokine in cell culture supernatant by Enzyme-linked immunosorbent assay (ELISA)

# ELISA assay


Lactobacillus
Activated-macrophage with culture medium
2-4 hours




Add antigen / culture supernatan

After TMB assay, measure colour change by microplate ELISA reader

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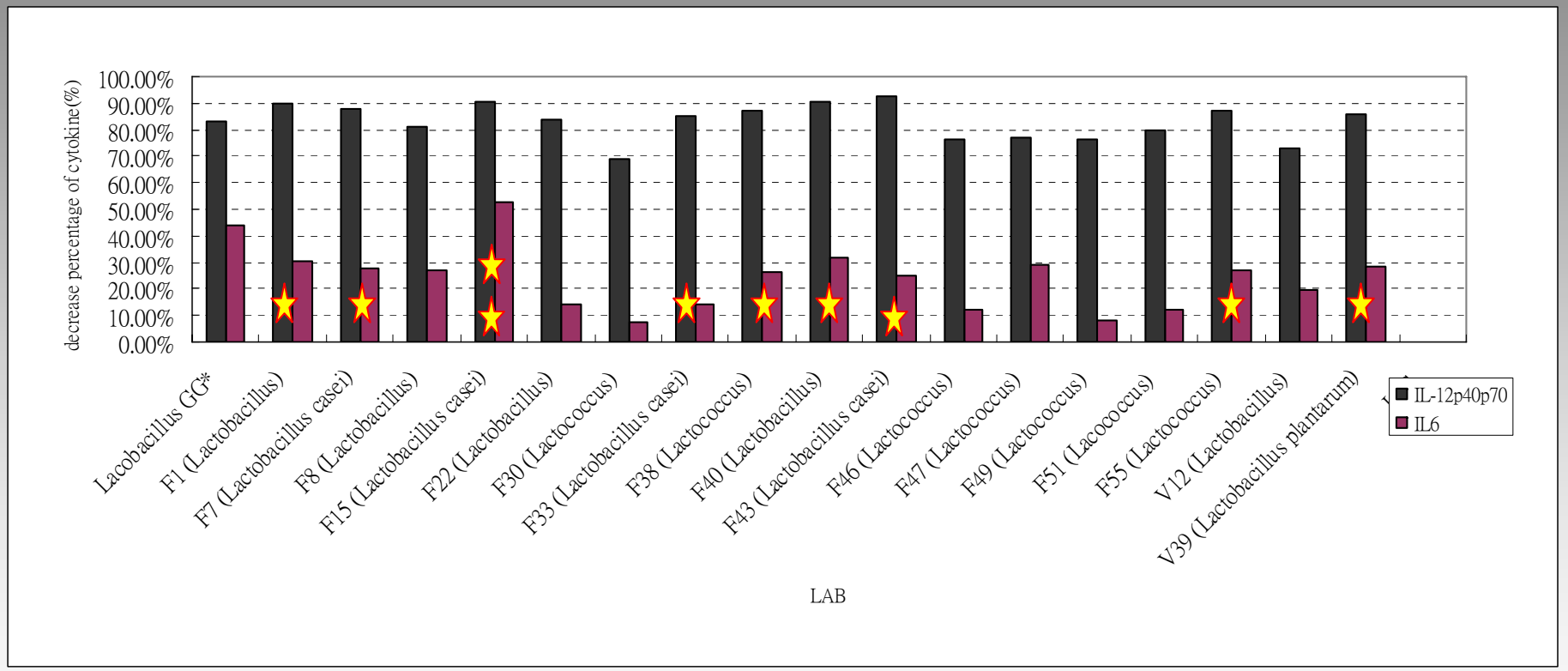
# 乳酸菌分離株與免疫的關係




(Wan Chen Wee, 2006)

# 乳酸菌分離株吸附與免疫的關係

(Wan Chen Wee, 2006)

| Isolated colony | LAB adherence count (CFU/cell)** | Determine to adherence*** | Th1 cytokine effect**** | Th2 cytokine effect**** |
|---|---|---|---|---|
| | | | IL-12p40p70 | IL-6 |
| Lacobacillus GG* | 45 | + | 83.09% | 44.23% |
| F1 (Lactobacillus) | 5 | - | 89.91% | 30.47% |
| F7 (Lactobacillus casei) | 25 | + | 87.92% | 27.94% |
| F8 (Lactobacillus) | 0 | | 81.41% | 27.09% |
| F15 (Lactobacillus casei) | 38 | + | 90.25% | 52.81% |
| F22 (Lactobacillus) | 1 | - | 83.63% | 14.49% |
| F30 (Lactococcus) | 8 | - | 69.14% | 7.22% |
| F33 (Lactobacillus casei) | 47 | + | 84.81% | 13.90% |
| F38 (Lactococcus) | 78 | + | 87.09% | 26.42% |
| F40 (Lactobacillus) | 3 | - | 90.30% | 31.71% |
| F43 (Lactobacillus casei) | 63 | + | 92.61% | 24.73% |
| F46 (Lactococcus) | 31 | + | 76.10% | 12.48% |
| F47 (Lactococcus) | 3 | - | 77.12% | 29.06% |
| F49 (Lactococcus) | 45 | + | 76.41% | 8.33% |
| F51 (Lacococcus) | 238 | ++ | 79.93% | 12.42% |
| F55 (Lactococcus) | 10 | - | 87.48% | 26.94% |
| V12 (Lactobacillus) | 245 | ++ | 72.93% | 19.35% |
| V39 (Lactobacillus plantarum) | 34 | + | 85.75% | 28.15% |

* ：Lactobacillus GG was probiotics strain and used as control in this study.

** ：Expressed as the average number of lactic acid bacteria adhering to Caco-2 cells, count is carried out on 20 randomized microscopic fileds.

***：The number lactic acid bacteria adhering to Caco-2 cells, which were lower than 15CFU/cells is thought as negative adhesion of lactic acid bacteria and shown as-；the number which were between 15 to 100 CFU/cells is thought as positive adhesion and shown as +；the number which were larger than 100 CFU/cells is stronger and shown as ++.

****：The data shown above were reduction percentage of cytokines. In this study, we turned immune system of mac[illegible]nto imbalance, that mean, we increased both Th1 and Th2 cytokine before, respectively, by using LPS. Higher the reduction percentage, that mean better th[illegible]imulation of Lactobacillus to macrophag[illegible] system.

# The End

Thank you for your patience