United Nations Nations Unies

International Seminar and Exhibition on Animal Feed Biotechnology

Using Bio-technology R&D New Feed Additive —— Transgenic Functional *Lactobacillus* Probiotics

利用生物技术研发新型饲料添加剂——转基因功能乳酸菌制剂

Wang Chun-feng

王春凤

Jilin Agricultural University

吉林农业大学

立论依据
Basis of making a point

The development of stockbreeding is the demand of adjustment of agricultral production structure of China.

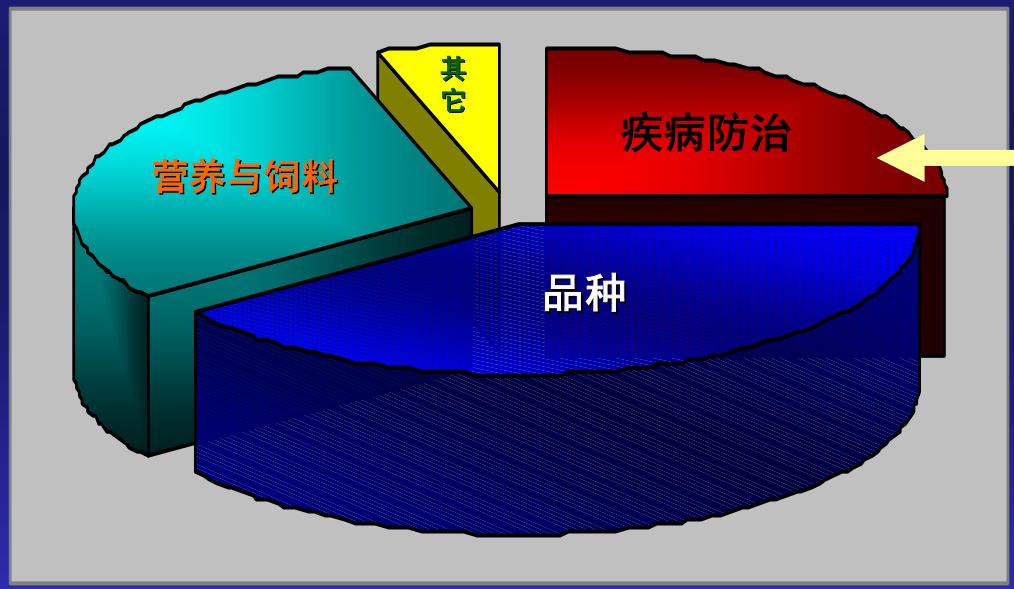
☆ 加快畜牧业发展。畜牧业是一个国家农业发展水平的重要标志。
☆ 畜牧业可以促进种植业，带动加工业，促进农业内部结构合理化和产业间的良性循环。
☆ 必须采取切实可行的政策措施，加快畜禽疾病防制体系建设，尽快把畜牧业发展成为一个大产业。

温家宝《当前农业和农村工作的主要任务》

－ 引自〖求是〗杂志第四期，2000年2月

畜牧业发展的需要

the Demand of Development of Stockbreeding



疾病是制约畜牧业发展的重要因素。

Disease is the major factor which limits the development of stockbreeding.

畜牧生产中各种科学技术的贡献率

the rate of contribution of all kinds of science and technology in stockbreeding industry

目前疾病控制的手段主要是传统疫苗和化学药物

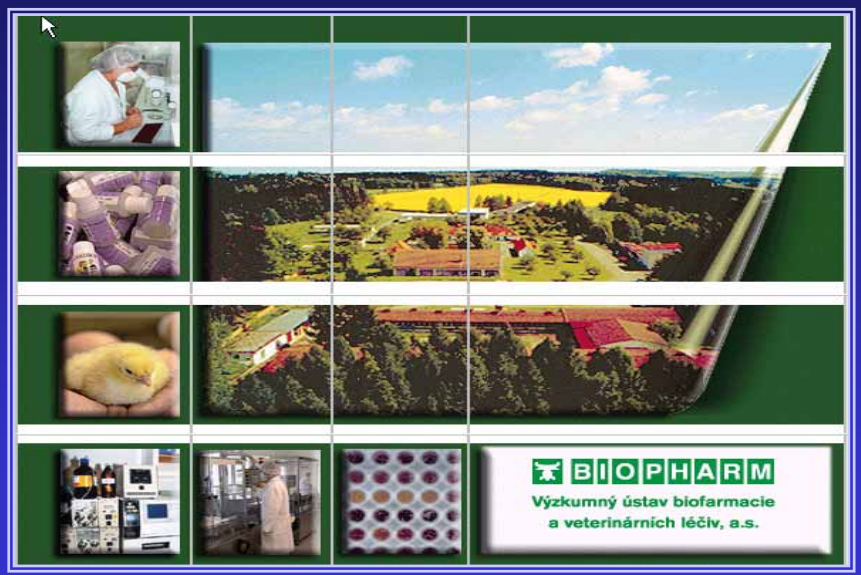
The present means of controlling diseases
is traditional vaccines and chemical drugs.

the Disadvantage of Traditional Vaccine——

1. The technics is complicate and the cost is high.

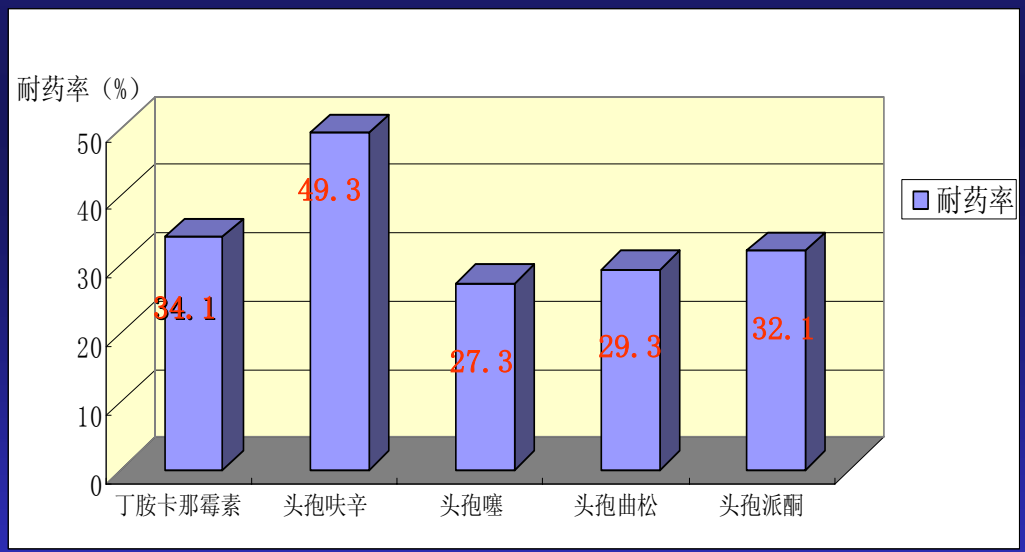
2. The storage and transportation need a special cool chain system.

3. All these limit the use of the third world.



化学药物的缺点
the Disadvantage of Chemical Medicine

A superbacteria was found by American scientists in feedstuff in 1992, which almost resisted all the present antibiotics.The magazine DISCOVERY claimed the antibiotics were going to the dead end.



北京积水潭中心医院对414革兰氏阴性菌与9种抗生素的耐药性研究结果 药学实践杂志 1997 年第 15 卷第 5 期

WTO的需要

The development of stockbreeding is the demand of WTO.

The residue of medicine is a major problem of restricting the export, though the output of livestock product of China comes out top in the world.

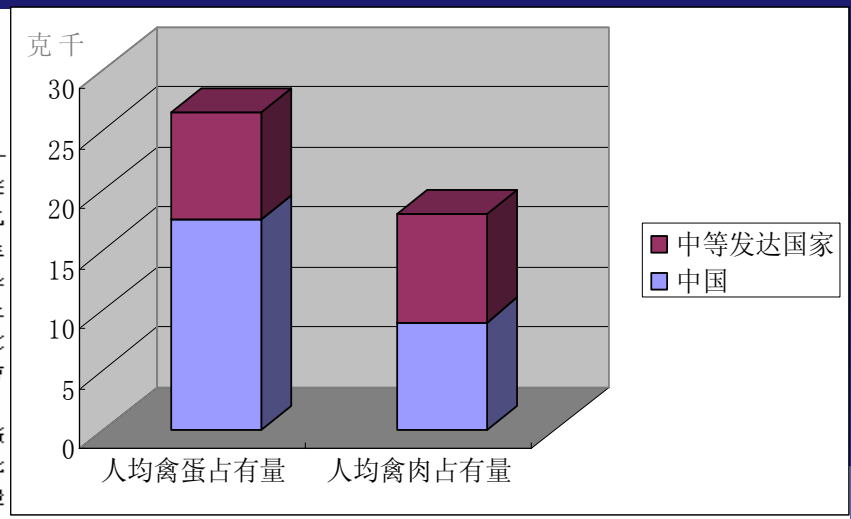
信息之窗

我国鲜蛋产量全球第一

改革开放以来，我国蛋禽饲养逐动发展。除了农户个体自给性养禽和千只以下小规模养禽以外，全国存栏在千只以上的规模蛋鸡场已达24.6万个，存栏蛋鸡6.26亿只。从1997年起，我国鲜蛋年产量已在2000万t以上，达到2125万t，与1987年590.2万t相比，10年增长260%，平均每年递增13.67；全国年产鲜蛋100万t以上的主产省有：山东、河北、江苏、河南、辽宁、湖北、黑龙江等七个省。其中山东、河北、江苏的产蛋量分别为388万t、301万t和219万t，成为12大产蛋区的前3位。

最近，据世界粮农组织公布的信息，1988年世界产蛋总量达到5300万t，其中我国鲜蛋产量为2220万t，比1997年又有新的增长，中国的产蛋已占到世界产蛋总量的41.9%。我国的鲜蛋产量不仅继续位居全球第一，同时鲜蛋的人均占有量也名列前茅。据世界粮农组织的信息，1998年，全球人均鲜蛋占有量8.9kg；而同一年中国人均鲜蛋占有量达到17.7kg，比世界人均占有量高出约1倍。

（引自《中国动物保健》1999年第5期）



我国禽蛋产量居世界第一位，占世界总产量的三分之一强；禽肉产量居世界第二位

WTO的需要

The development of stockbreeding is the demand of WTO.

畜禽产品出口的最大障碍

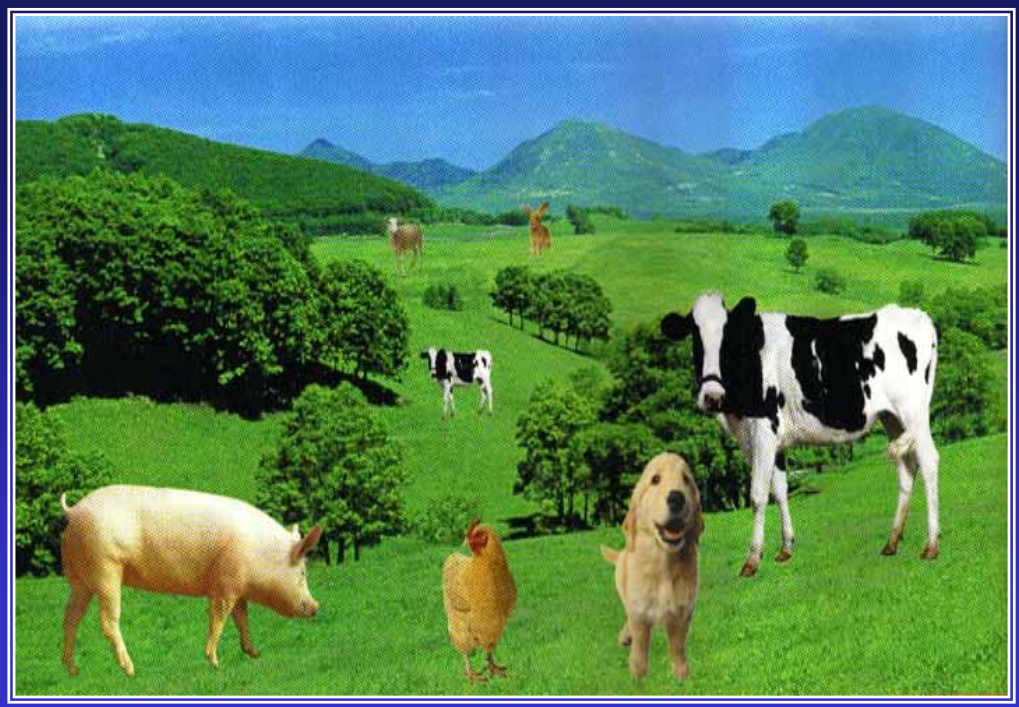
——药物残留问题

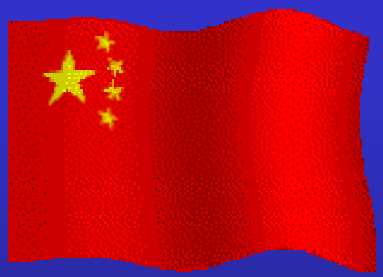
中国进入 WTO 后，中国畜禽产品是否能大量出口，最大障碍是畜禽产品中药物残留超标问题。多年来，我们对畜禽产品中对人体健康有害的药物残留重视不够，缺乏监督、监测及管理，导致畜禽产品外销不畅，1999 年我国出口肉类 402277 吨，蛋类 339109 吨，奶类 133628 吨，水产类 776102 吨。出口肉类占全国肉类总产品的 0.8%，蛋类、奶类、水产类出口比例也非常小。大量畜禽产品只能内销，其主要原因就是畜禽产品中药物残毒超标所造成的。例如，前些年出口日本的肉鸡，由于“克理粉”超标，被退货和中断出口；出口法国蜂蜜由于农药“杀虫脒”残毒超标而被退货；1998 年 4 月出口香港活猪，内脏中含“盐酸克伦特罗”导致香港 17 人中毒，被香港东方日报暴光等；另外，畜禽产品中含有某些抗生素，人吃了还可以引起过敏反应。最近世界卫生组织呼吁限制对牲畜使用抗生素，而我国畜禽生产中大量滥用抗生素及禁用的安眠胴类、激素类药，都影响畜禽产品的质量，降低我国畜禽产品在国际市场上的竞争力，应引起我国政府部门、广大养殖场(户)及饲料场家的高度重视。

如何降低畜禽产品药物残留和生产出环保型的放心畜禽产品是我们亟待解决的问题

How to solve these problems ?

It is a hotspot for us to study a new type of vaccine or additive , which is safe , effective , non-rudimental , low-cost ,straight edible.





绿色环保型——
畜禽基因功能乳酸菌的研究与应用

Study and Application of Genetic Functional *Lactobacillus* Probiotics

目的和意义

Purpose and Significance

（1）消化道共生乳酸菌受体菌株的意义

the significance of symbiotic *Lactobacillus* as a recipient bacteria strain in digestive tract

（2）构建乳酸菌非抗性表达载体的意义

the significance of constructing a non-antibiotic expression vector of *Lactobacillus*

（3）基因功能乳酸菌的应用前景

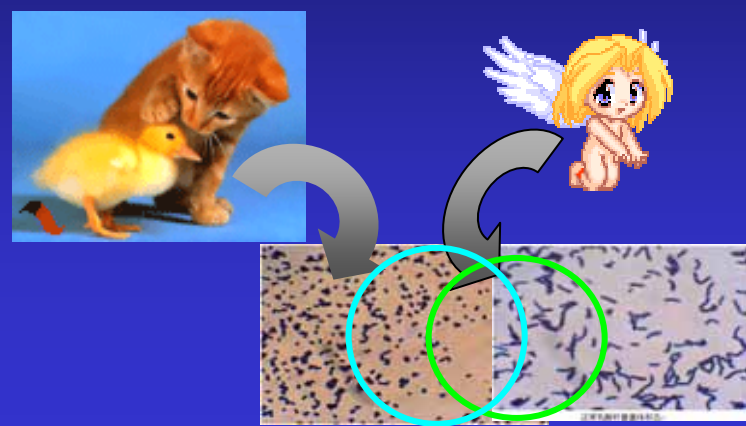
the perspective of genetic functional *Lactobacillus*

乳酸菌的生物学特性

the Biological Character of *Lactobacillus*

Since *Lactobacillus* was found by Pasteur in 1857, many other kinds of *Lactobacillus* were separated and identified and studies are developing quickly.

As an important member of enteric normal bacteria of human being and animals, *Lactobacillus* has many beneficial functions, such as improving immunity and inhibiting pathogenic micro-biology and so on.



乳酸菌的分子遗传学概况

the General Character of Molecular Genetics of *Lactobacillus*

Comparing to the other engineering recipient bacteria strains, the step of studying on *Lactobacillus* hangs behind.

基因功能乳酸菌制剂特点

the Character of Genetic Functional *Lactobacillus* Probiotics

- ※ security
- ※ simplicity
- ※ efficiency
- ※ universality

研究内容
the Major Research Content

（一）、载体的构建

the construction of vector

1. 非抗性高效分泌型乳酸菌表达载体的构建；

non-antibiotic , secretory , high-effective expression vector of *Lactobacillus*

2. 安全高效植物表达载体的构建；

safe, effective expression vector of plant

3. 高效真核多价DNA疫苗表达载体的构建；

effective , multi-valent, DNA expression vector of eukocyte

（二）、免疫调节因子的筛选、克隆与装配

to screen, clone and assemble the immune regulative factor

（三）、重要疾病抗原基因的筛选克隆

to screen and clone some antigen genes

研究内容
the Major Research Content

（四）、保护性抗原基因的表达及表达产物的检测

to detect the expression of protective antigens

（五）、各种疫苗的临床免疫效果实验

the experiment of clinical efficiency of all kinds of vaccines

（六）、检测基因功能乳酸菌回归动物肠道后的生物学效应及其演替规律

to detect the rule of inhabitation and succession of *Lactobacillus* after returning the digestive tracts of animal

（七）、进行中试实验及其产业化生产

the trial period and industrial production

技术路线 the Route of Technology

从健康动物消化道中筛选、鉴定共生乳酸菌受体菌株

1. ThyA缺陷型受体菌株的筛选
2. Nisin抗性的受体菌株的筛选
3. B-gal缺陷型受体菌株的筛选

1. 增菌培养
2. 选择性培养
3. 菌种鉴定

ThyA/Nisin/B-gal为选择压力乳酸菌非抗生素抗性载体表达系统的构建

ThyA基因、Nisin基因、B-gal等基因的PCR扩增

绿色荧光蛋白基因（GFP）的PCR扩增

将ThyA、Nisin、B-gal等基因与GFP基因进行连接，形成非抗生素抗性且易于检测的双标记基因

选择高分泌的乳酸菌启动子-分泌信号肽基因，并克隆、串联与组装并检测

乳酸菌的基本质粒载体pW425e的酶切，以去除抗生素抗性基因

ThyA（Nisin/B-gal）-GFP等基因与载体大片段、分泌信号肽的连接、转化、鉴定、检测

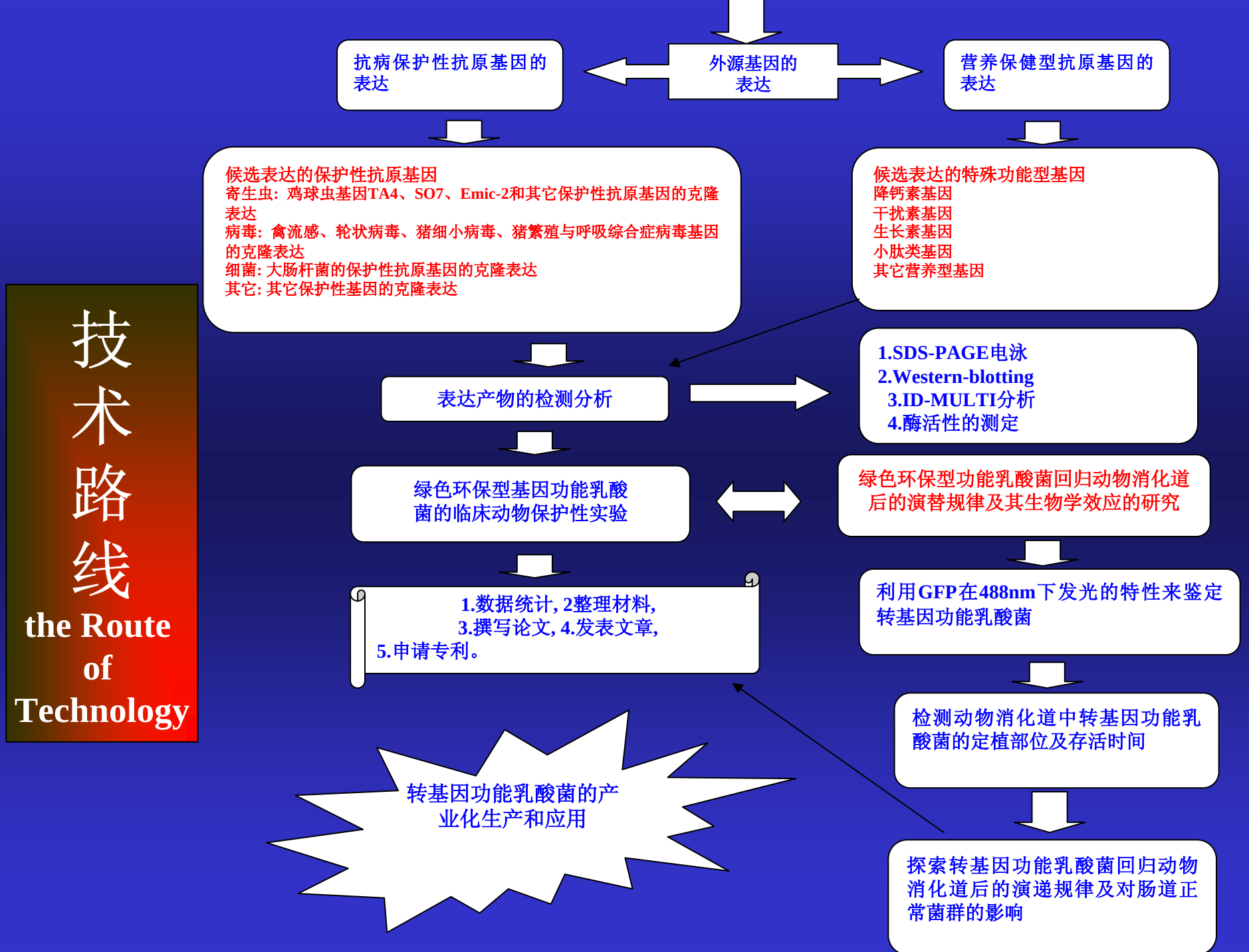
加入表达调控元件

将此三套非抗性表达载体分别转化入相应的乳酸菌受体菌株中，并进行稳定性研究

以一个基因为例，检测载体的有效性

. GFP的荧光的检测（488nm）
. Northern-blotting
. SDS-PAGE分析
4. Western-blotting
5. ID-MULTI分析
6. 免疫细胞化学分析

获得最佳的可调控的易于检测的乳酸菌高效分泌非抗性表达载体技术平台，为外源功能基因的表达奠定基础


技术路线
the Route of Technology
外源基因的表达
抗病保护性抗原基因的表达
营养保健型抗原基因的表达
候选表达的保护性抗原基因
寄生虫：鸡球虫基因TA4、SO7、Emic-2和其它保护性抗原基因的克隆表达
病毒：禽流感、轮状病毒、猪细小病毒、猪繁殖与呼吸综合症病毒基因的克隆表达
细菌：大肠杆菌的保护性抗原基因的克隆表达
其它：其它保护性基因的克隆表达
候选表达的特殊功能型基因
降钙素基因
干扰素基因
生长素基因
小肽类基因
其它营养型基因
表达产物的检测分析
1.SDS-PAGE电泳
2.Western-blotting
3.ID-MULTI分析
4.酶活性的测定
绿色环保型基因功能乳酸菌的临床动物保护性实验
绿色环保型功能乳酸菌回归动物消化道后的演替规律及其生物学效应的研究
1.数据统计，2整理材料，
3.撰写论文，4.发表文章，
5.申请专利。
利用GFP在488nm下发光的特性来鉴定转基因功能乳酸菌
检测动物消化道中转基因功能乳酸菌的定植部位及存活时间
探索转基因功能乳酸菌回归动物消化道后的演递规律及对肠道正常菌群的影响
转基因功能乳酸菌的产业化生产和应用

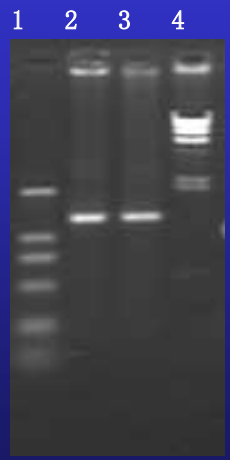
Some Studying Projects

- 1. “Studies on the gene engineering ***Lactobacillus*** vaccine against Rotavirus ” , the National Science and Technology Bureau of china 863 project , 1.5 million, 2003---2005
- 2. “Studies on the localization and biological effect of the gene engineering *Lactobacillus* returning to the chicken intestine” , the national nature science fund , 0.22 million, 2003---2005
- 3. “Studies on the immunity mechanism of gene engineering *Lactobacillus* vaccine” ,the outstanding young people fund of Jilin province science and technology bureau , 0.1 million, 2003---2005
- 4. “The development and application of the gene engineering *Lactobacillus* vaccine” , the significant agricultural item of Jilin province science and technology bureau, 0.1 million, 2003---2005
- 5. “The development of gene engineering *Lactobacillus* vaccine against *Eimeria tenella* of chicken” ,the significant scientific and technological item of the national education bureau, 0.1 million, 2003---2004
- 6. “The construction of non-antibiotic, secretory and high-effective expression *Lactobacillus* vector”, the youthful teacher startup fund of Jilin agricultural university, 0.03 million , 2003---2005
- 7. The national Postdoctoral science sustentation fund, 0.01 million , 2002---2004
- 8. The national Postdoctoral fund , 0.06 million , 2002---2004

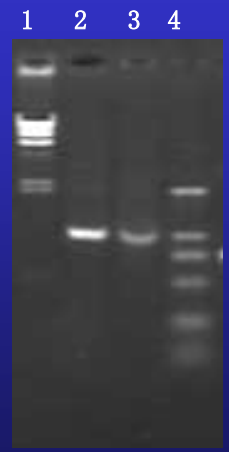
Some Periodical Accomplishment

1. successfully gained a *Lactobacillus* recipient strain absent of genes,such as ThyA gene 、 β-Gal gene
2. successfully constructed a non-antibiotic expression ,secretory vector with ThyA/β-Gal gene as selective pressure
3. successfully expressed some protective genes of livestok and avian ,such as S07,TA4 and VP4,VP6,VP7 and NSP4 genes of Rotavirus
4. The result of animal experiments showed Newly-born chicken were feed with oral solution of engineered *Lactobacillus*, and infected with sporulated ocysts (5×10^4) of *E. tenella* two weeks later. Test chicken were evaluated by scoring cecal lesions, weight gain, amount of oocysts of *E. tenella* in cecum and feces and ACI to determine the level of protection. Results showed that the engineered *Lactobacillus* had significant effects on protection of chicken against *E. tenella* (ACI: 163.40).

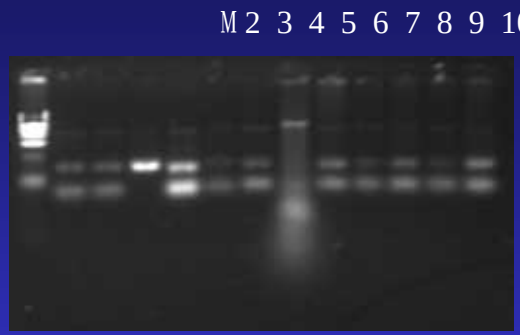
Some experiment results



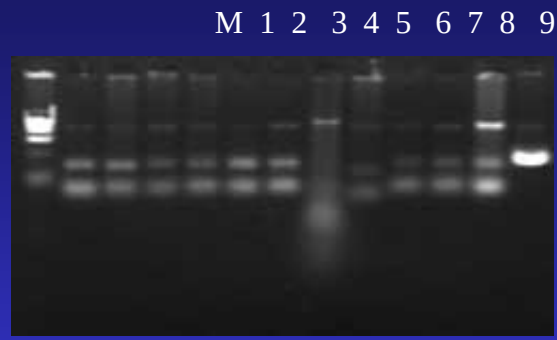
VP4 gene of Rotavirus was cloned by PCR
1. DL2000 Marker; 2.3：VP4扩gene;
4. λ DNA/ EcoRI + HindIII Marker



VP7 gene was cloned by PCR
1.DL2000 Marker; 2.3：VP7扩增的基因;
4. λ DNA/ EcoRI + HindIII Marker

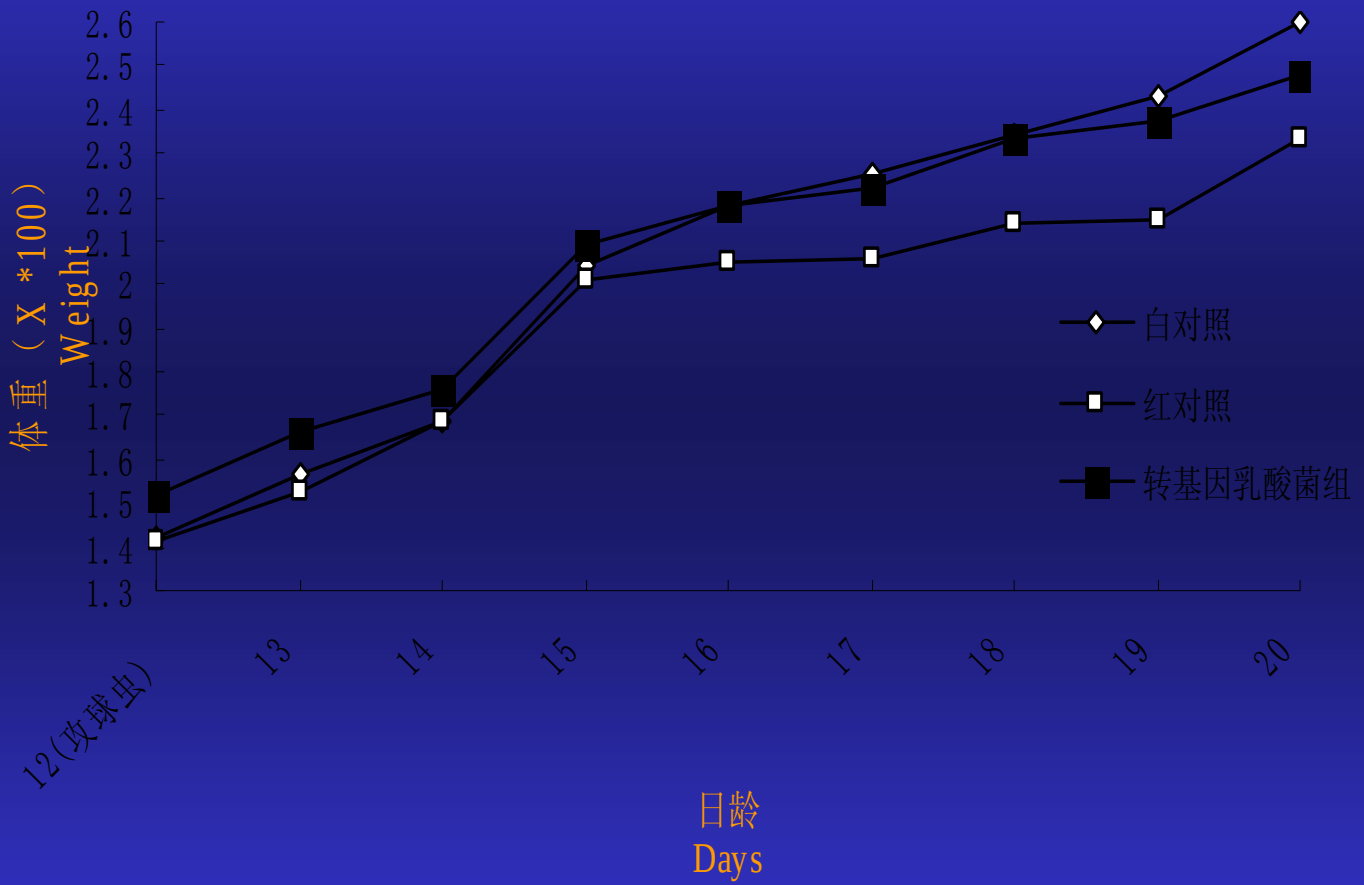


VP4质粒的提取



VP7质粒的提取

Some experiment results

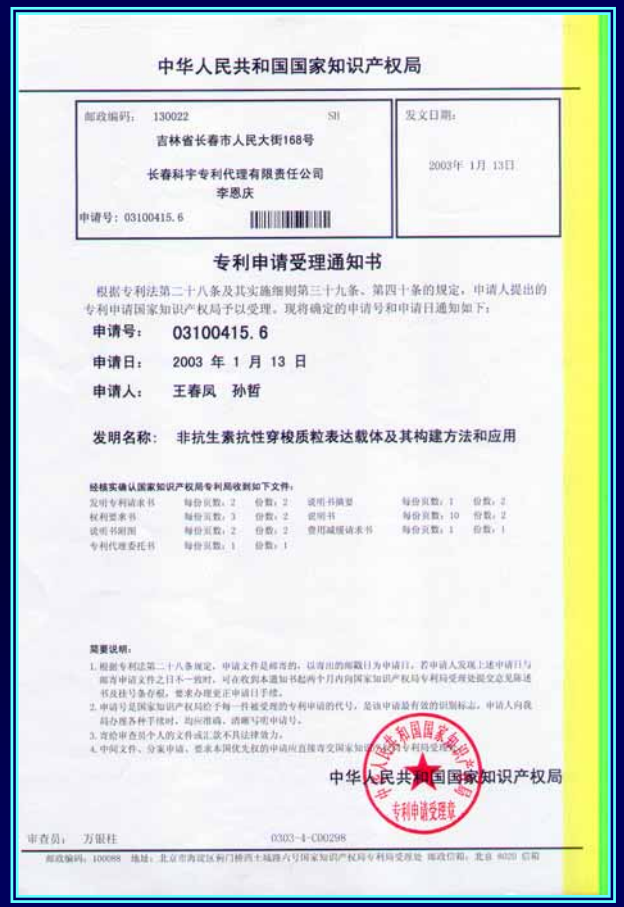


专利和知识产权

Acquired Patent

乳酸菌高效分泌型载体表达系统的构建方法及技术成果已经申请发明技术专利。

The method of construction and the production of technology of high – effective, secretory expression vector of Lactobacillus have Acquired Patent



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申请号：03100415.6

发文日期：

2003年 1月 13日

专利申请受理通知书

根据专利法第二十八条及其实施细则第三十九条、第四十条的规定，申请人提出的专利申请国家知识产权局予以受理。现将确定的申请号和申请日通知如下：

申请号： 03100415.6

申请日： 2003 年 1 月 13 日

申请人： 王春凤 孙哲

发明名称： 非抗生素抗性穿梭质粒表达载体及其构建方法和应用

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权利要求书	每份页数：3	份数：2	说明书	每份页数：10	份数：2
说明书附图	每份页数：2	份数：2	费用减缓请求书	每份页数：1	份数：1
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the Published Articals

1. **1. Wang Chunfeng, Qin Zerong, Sun zhe. Effect on chicken weight by feeding the gene engineering *lactobacillus* vaccine transformed by Eimerria tenella SO7 gene. Chinese Bulletin of Husbandry and Veterinary, 2002,33(6)**
2. **2. Wang Chunfeng, Qin Zerong, Sun zhe. Cloning and Sequence Analysing of Bulgaria *lactobacillus* β -Gal gene. Chinese Bulletin of Agricultural Biological Technique, 2003,11(2)**
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市场需求分析

the Analysis on the Demand of Market

1. The probiotics of transgenetic *Lactobacillus* maybe substitute the antibiotics and traditional vaccines,partly or even wholly,whose demand of market could be very large.
2. *Lactobacillus* is foof-grade and feed-grade bacteria strain,which has immeasurable perspective of market.

展　望
Perspective

With the realization of physical and health care functions of *Lactobacillus* and the development of technology of molecular biology,all kinds of food-grade and feed-grade *Lactobacillus* strains and other engineering bacteria strains with will come forth, which is to bring infinite economic,social and ecological benefit.The *Lactobacillus* engineering will more and more splendent in the 21th century.


Thanks !