# An Effective Cloning, Heterologous Expression and Physiological Activity in *Lactococcus lactis* NZ9000 of Catalase Gene from *Escherichia coli* DH5α

**Abstract** In order to clone active Catalase gene from bacteria, we introduced a method of shotgun integrating specific sceen from *Escherichia coli* DH 5α. Genome DNA was extracted from *E.coli* DH5 and partially digested with *Sau*3AI. Then some fragments more than2.26kb were collected and ligated with T4 DNA ligase into the *Bam*HI-cleaved plasmid pUC18 and transformed competent *E. coli* TG1 cells. The transforments were incubated ananaer obically on brain heart infusion (BHI)containing tannic acid.The method could detect catalase activity and screened the catalase-positive clones. And the catalase protein can be identified by SDS-PAGE. The results showed that the recombinant plasmid pUC18-kat was constructed successfully by PCR identifikation and restriction enzyme digestion.Open reading fram from DNA Sequence DH5a katE. The sequence long is 2262nt and coding protein are 753 Amino Acids, Molecular Weight 84198.72 Daltons. This method is simple, and possessed of popularization and application value.It would lay a fundation for cloning an active catalase later. Furtherly, In this study, the fragment of 2,262 bp catalase gene *katE* was cloned into the expression vector pQE30 and transformed into *Escherichia coli* M15, and KatE protein was expressed after the induction with Isopropylthio-β-D-galactoside. The KatE protein was separated on SDS-PAGE and recovered using a His-tag affinity . New Zealand white rabbits were immunized with the purified protein to harvest polyclonal antibodies .As *L. lactis* has no catalase, *katE* was inserted into *Escherichia coli - L. lactis* shuttle vector pMG36e and electrotransformed into *L. lactis* NZ9000. The expression of the KatE protein was confirmed by SDS-PAGE analysis and Western blot. Further experiment demonstrated that the expression of the KatE gene in *L. lactis* NZ9000 is able to produce active catalase that can provide efficient antioxidant activity. Additionally, to understand the import of catalase katE gene of Lactococcus lactis on the body physiological changes of immune function in mice.Enzyme-linked immunoassay (ELISA) was the blood of mice, IgG, IgE, and CD4 and CD8 levels, find out whether the difference between Grouping more mice eating the recombinant L.lactis NZ9000 and other groups (recombinant *E.coli* DH5α and L.lactis NZ9000, E.coli DH5α and saline) The experiments showed that recombinant L.lactis NZ9000 was significantly higher than the other on IgG concentrations, the difference was significant; IgE of CD4 of CD8 levels are no significant difference. Mice after the intake of recombinant L.lactis NZ9000 increased IgG levels explain recombinant L.lactis NZ9000 regulatory role of humoral immunity in mice; IgE level did not change CD4, CD8 levels were also no change, suggesting that there is no significant effect on the body of cellular immunity in a short time.

**Key words:** an effective method, active catalase gene, cloning and screening, tannin resistance

## Introduction

*Lactococcus lactis* (*L.lactis*) is the model organism of lactic acid bacteria which are widely used for the dairy industry and other food fermentations but also for an increasing number of biotechnological applications. During industrial processes, this bacterium has to cope with various

stresses including low pH, high temperature, osmotic shock, metal stress, acidity, and oxygen ($O_2$) as well as carbon limitation (Rochat *et al.*, 2012; van de Guchte *et al.*, 2002).

Catalases are antioxidant metalloenzymes that disproportionate $H_2O_2$ to water ($H_2O$) and $O_2$ during aerobic growth (Abriouel *et al.*, 2004). And when the concentration of $H_2O_2$ is low, catalase can catalyze the oxidation of electron donors, such as ethanol or phenols. Therefore, in microbiology, the catalase test is also used to differentiate between bacterial species in the lab (Percy, 1984). Catalase is a very highly conserved enzyme that is ubiquitous in bacteria, fungi, plants and animals. Bacterial catalases are widespread in aerobes such as *Escherichia coli* (*E.coli*). And Catalases are classified into three major families: Mn-catalases, catalase-peroxidases and monofunctional catalases, the monofunctional catalases are the best characterized heme-containing enzymes (Arockiaraj *et al.*, 2012).

Catalase is used in the food industry for removing hydrogen peroxide from milk prior to cheese production and preventing food from oxidizing ((Boucher, I.et al.2002, Martirosyan A.O., et al. 2004 ,Jacek S, Loewen P C.2002). Additionally catalase has also been used in the aesthetics industry.

It was proved that gallate decarboxylase activity was elevated in the presence of tannic acid in *S. gallolyticus* (Chung K T et al.1998). It demonstrated that some *enzyme activity could make the* organism with a selective advantage over *S. bovis* when grown in the presence of tannins（Engesser, D. M et al 1994).

At present, many methods are used to clone genes. Such as they are got by genome bank, PCR,shotgun, RT-PCR and so on (Sambrook, J.et al. **1**989). To clone the active catalase from bacteria, an effective approach was assayed (Chung K T et al.1998). In this study, we cloned the active catalase of *E,coli* DH5a. We used methods including cloning, vector constructed, sequencing, and expression. We also screened selected the activity of catalase by the presence of tannic acid in recombinant strains in vitro. Additionally, to understand the import of catalase katE gene of *Lactococcus lactis* on the body physiological changes of immune function in mice. The levels of IgG, IgE antibody and CD4 and CD8 cells in the blood of experimental animals were examined by enzyme-linked immunoassay (ELISA) for laying the foundation of further study recombinant *L lactis.*

## Materials and methods

A. DNA manipulations and plasmid construction

Table 1.The bacterial strains and plasmids used in this study

| Bacterial strain or plasmid | Description[a] | Source or reference |
|---|---|---|
| Bacterial strains | *E. coli* Top10,TG1,DH5a Plasmidless *Lactococcus lactis* NZ9000 | Invitrogen and this lab. |
| pMD18-T | Cloning vector, $Ap^r$, 2.692 kb, pBluescript SK+/ | New England Biolabs |
| pET-28a(+) | Expression vector, $Ap^r$, 5.369 kb | Gibco BRL |
| pTkatE | MD18-T containing 2.5-kb *Bam*HI insert *of catalaseE* DH5a genomic fragment | This work |
| pVE3655 and pMG36e | PET28a(+) containing 2.262-bp *Xba*I-*Sph*I PCR product insert of catalase gene, plasmids pVE3655 and pSEC(donated by Dr.Gruss) | This work |

The table shows the vectors and bacteria strains for this study.

## Cloning and transformation.

In order to clone the catalase gene, total genomic DNA was isolated and purified with DNA purfing kit (Qiagen). And it was partially digested with *Sau*3AI, and the DNA fragments of 1.5 to 2.5, 2.5 to 3.5, 3.5 to 5, and 5 to 7 kb were purified from the agarose gel using the QIAEX II kit (Qiagen). DNA fragments ligated with T4 DNA ligase (Qiagen) into the *Bam*HI-cleaved plasmid pBluescript SK+/− were used to transform electrocompetent *E. coli* Top10 cells **(**Sambrook, Jet al. 1989). The gene library was constructed that of about 1,500 clones. The transformants were screened for catalase activity on brain heart infusion (BHI) containing tannic acid (BHI-tannic acid medium) and ampicillin (150 μg/ml), which is the same medium used for screening for tannase activity (Chung K T， et al.1998). This medium was used as a plate-screening assay which could detect catalase activity when cloning into catalase-positive bacteria such as *E. coli*. First, the plates were incubated anaerobically for 2 days to allow the colonies to grow and to prevent darkening of the medium; the plates were then kept aerobically for a further 24 h at 37°C to induce the catalase gene (Hikmate A et al.2002). A zone of clearing surrounding the colonies which developed during aerobic incubation, indicated tannic acid degradation as a result of catalase activity. Plasmid DNA (pHA01) of one clone was isolated using the Qiagen Midiprep plasmid purifikation kit (Qiagen), and the insert was sequenced bidirectionally at GATC Biotech (Konstanz, Germany) using M13 universal oligonucleotide primers and custom primers. Plasmid DNA (pMD18-T-kat) exhibiting presumptive catalase activity on BHI-tannic acid medium was

shown to harbor a 2.26kb DNA fragment insert. The expression vector (pET28a(+)) was constructed as followed

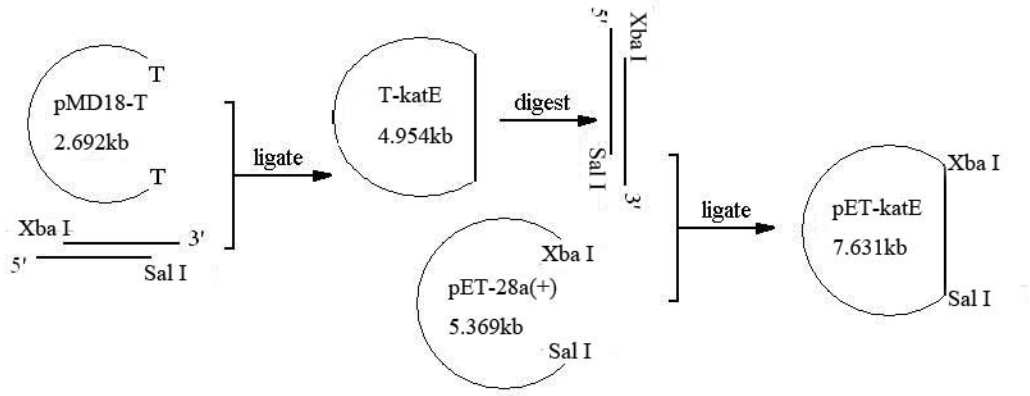


Fig. 1 Construction of recombinant plasmid pET-katE

*E. coli* was transformed by the method of electroporation (Powell I Bet al.1988) which was pre-digested with SalI and XbaI restriction enzymes. After ligation, the reaction mixture was employed to transform DH5α competent cells, and the recombinant plasmid having a size larger than pET28a was then screened out. it was confirmed that the cloned DNA fragment of 2.2 kb was the katE gene and the construction of expression plasmid pET 20b/katE had thus been accomplished.

**Amplification by PCR and oligonucleotide synthesis**.

PCRs were performed with a Perkin-Elmer Cetus (Norwalk, Conn.) apparatus using *Taq* DNA polymerase (Promega) as recommended by the manufacturer. Oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems, San Jose, Calif.). A 2262-bp fragment was PCR amplified from the $pUC_{kat}$ matrix. The oligonucleotides used were XbaI-K-UP：5’-TCTAGAATGTCGCAAKATAACGAAAAGAACCCAKATCAGC-3’ (oligo 1) for the coding strand and SalI-K-Down：5’-GTCGACTTACGCCGGGATTTTGTCAATCTTAGGAATGCGTG-3’ (oligo 2) for the complementary strand. The reaction contions 94℃ for 3 min, then 30 cycles of 94℃ for 0.5 min, 50℃ for 50 sec, 72℃ for 1 min, followed by a final extension of 5 min at 72℃. The amplified fragment was purified using the QIA quick PCR purifikation Kit (QIAGEN Inc), and digested with *EcoR* I and *Not* I. The resulting fragment was respectively ligated into the contained a T7 promotor plasmid pET28 digested with *EcoR* I and *Not* I. This DNA fragment was then cloned on pET28 vector in *E. coli* TG1, resulting in pET28 kat. And *katE* was Also inserted into *Escherichia coli - L. lactis* shuttle vector pMG36e and electrotransformed into *L. lactis* NZ9000.

Protein detection of catalase. In order to assay the production as well as the expression of catalase induced by IPTG in *E. coli* BL21（DE3）cells, the cell extracts by ultrasonic crashing and supernatants of the different *E. coli* recombinant clones were examined by SDS-PAGE (Hikmate Abriouel,et al.2004, Bernhard T et al 2004). Preparation of cellular and supernatant protein fractions of L. lactis was confirmed by SDS-PAGE analysis and Western blot. For fractionation

between cell and supernatant fractions, 2 ml samples of nisin-induced L. lactis cultures were centrifuged for 5 min at 6000 *g* at 4 □. Protein extracts were then prepared as previously described (Le Loir et al., 1998). Further experiment demonstrated that the expression of the KatE gene in *L. lactis* NZ9000 is able to produce active catalase that can provide efficient antioxidant activity.

**Bacterial cultivation and the sample prepared**

Microbial strains of recombinant Lactococcus lactis and normal NZ 9000 were obtained from the lab of Dr. Li (Central of biopharmaceutical engineering in Zhaoqing university, China). They were cultured in 100 mL of MG17 broth overnight at 30 °C and induced by Nisin for 2-3 h. *E.coli* DH5αcultured in 100 mL of LB broth overnight at 37 °C. The count of bacteria was obtained by plate count method under a microscope. The bacterial cells were centrifuged for 10 min at 3,000×g and washed twice with the sterilized PBS (pH 7.4). Finally, bacteria were resuspended in GM17 medium (pH 7.4) under gentle vortex mixing. The samples were prepared for oral of the mice.

**Sequence alignment and analysis of the *kat* gene.** The cloned catalase gene was sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Sequence alignment and analysis of the *kat* gene were performed by DNAstar software.A number of *kat* homologs have been identified and characterized in many different bacteria, such as *Bacillus subtilis subsp, Escherichia coli O157:H7 str, Escherichia coli str. K-12 substr, Pseudomonas syringae , Salmonella enterica subsp, Yersinia pestis, Staphylococcus carnosus subsp, hay bacillus* . Alignment of sequences from different clones and analysis of the sequence for ORFs were carried out with DNAstar package. Sequence similarities were analyzed with the BLAST and FASTA programs.

**The mice fed with the samples**

Female BALB/c and C57BL/50 mice (7–8 weeks old) were gently provided by Center of experimental animals in Sunyat University (China) and 5 groups were divided for 10 each one group.This work was performed according to the international guidelines for animal laboratory care. Noninduced cultures and *L. lactis* NZ strains harbouring the control plasmid pVE3655 (Table 1) were used as negative controls. Groups consisting of at least 10 mice each were inoculated intragastrically with 0.5 ml of sampling fluid of recombinant *L.lactis* and recombinant *E.coli* (test groups) and of normal *L.lactis* and *E.coli* (control groups). The animals were kept in separated cages and had free access to an autoclaved pelleted diet and sterile water during the experimental period. Four weeks after inoculation, the animals were killed under ether anesthesia and blood of the experimental mice obtained for microbiological and immunological studies. The spleen was gently disrupted through a nylon mesh and the cells analyzed for *in vitro* cellular proliferative response and T and B lymphocyte subset profile in response to *H. pylori* antigens

**Immune function analysis of experimental mice.**

Spleen cells (1.0 x 106 cells in 900 _l of RPMI 1640 medium containing 10% heat inactivated fetal calf serum, 200 mM L-glutamine and 50 _g/ml gentamycin) from the experimental mice were cultured for 48 hr at 37°C in 24 well microplates (Nunc). The cells were stimulated with 100ul of sonicated suspension of each *H. pylori* antigen preparation at a final concentration of 4ng/ml, stained with phycoerythrin-conjugated antibodies anti-IgG anti-CD4_, anti-CD8 (Sigma Chemical Co.,St. Louis, Missouri, USA). The percentages of CD4_ (helper),CD4/ CD8_ (suppressor) T and B cells

were determined by the kit (Becton Dickinson) using Cell Quest software. A total of 104 viable

cells were analyzed after cell debris had been gated out. The results were expressed as percentage of blast cells (CD4_, CD8_ T and B cells) or lymphocytes (CD4_CD25_ T cells). ELISA for katE-specific IgG and IgE antibodies was performed by porcine anti-rotavirus A IgG and IgE antibodies as described in the manufacturer's instructions. but the secondary antibody was exchanged by peroxidise-labelled goat anti-porcine IgE (Thermo Fisher Scientific, Bonn, Germany) at a dilutionof 1/10 000. The absorbance of each sample was measured at 450 nm.

**Salt stress**

Salt stress experiments were performed in TYG broth. Early-exponential-phase cultures were harvested and resuspended in TYG broth containing 20% NaCl and incubated at 30°C for 8h, plating serial dilutions at 2h intervals.

**Statistical Analysis.** The results were presented as median percentage values and analyzed by the two-tailed Mann-Whitney T test. The level of significance was set at $P< 0.05$.

## Results

### Genome DNA extraction

*E. coli W3110* genome DNA was extracted and identified by 1percent agarose (see Fig 2)

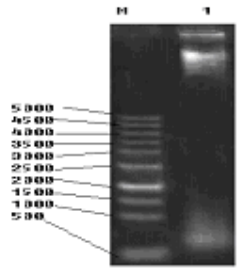


Fig 2 . DNA electrcphoresis from E. coli
M:Marker, 1 Genome DNA

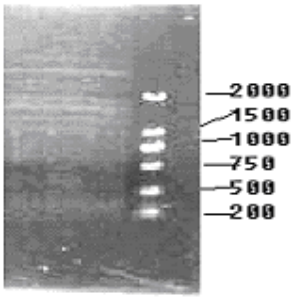


Fig 3 . Electrophoresis from E. coli total genomic DNA partially digested with Sau3AI
M:Marker;1:genomic DNA partially digested with Sau3AI

### Positive clone selection

The positive clones more than 1500bp molecular weight with catalase activity on brain heart infusion (BHI) containing tannic acid and ampicillin for screening for tannase activity after transformation were selected ( see Fig 4,5).

Fig 4 a control plate without catalase activity

Fig 5 a plate-screening assay with catalase activity

### Kat gene identification

The kat gene was identified correctly by PCR and restrictive enzymes *Xba*I / *Sal* (see Fig 6,7). The recombinant *L.lactis containing kat gene* was identified by PCR and restriction enzyme

analysis.when the recombinant plasmid was transformed into *E.colis* by electrophoration. The results from the Figure 7 and 8 showed that the kat gene isolated in the E.*coli* was correct.

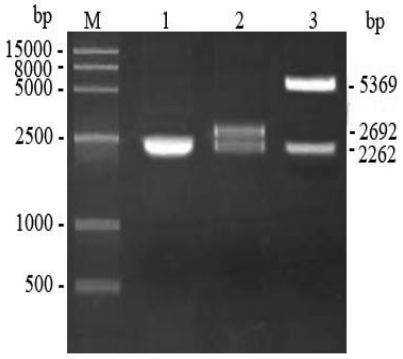


Fig.6 Identifikation of PCR product of *katE*, recombinant plasmid T-katE and pET-katE
M: DNA marker; 1: PCR product; 2: T-katE digested with *Xba*I / *Sal* I; 3: pET- katE digested with *Xba*I / *Sal* I.

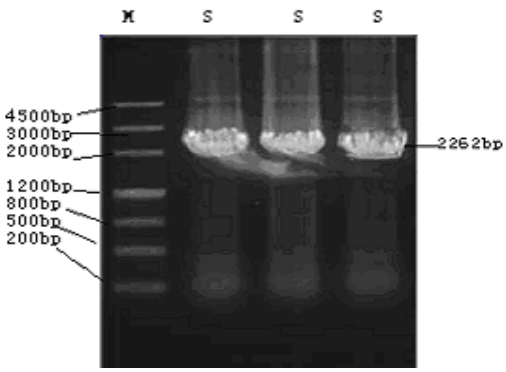


Fig 7. kat gene identifikationby PCR
M: Marker, S: product by PCR

***Expression of kat gene in E.coli***

The recombinant *E.coli* were incubated and induced by *IPTG* in LB medium for 6 hours. The KAT protein was identified by SDS-PAGE and Western-blot with the antibody prepared from the rabbits immuned by recombinant KAT protein (prepared in our laboratory). The results confirmed that KAT protein had been expressed obviously in *E.coli*. The molecular weight is 84.2kD. The plasmid containg kat gene was called pET-katE. And it was proved by Western-blot.The quantity of expression was about 10 mg/ml (Figure8,9).

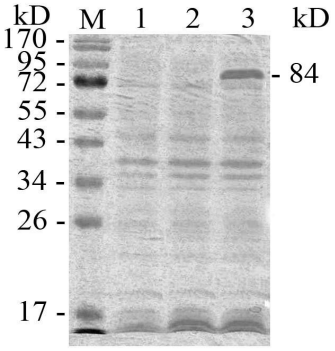


Fig. 8 SDS- PAGE analysis of KATE expressed in *E. coli* BL21（DE3）
M: protein marker; 1: BL21(DE3) with IPTG induction; 2: pET-katE clones transformed *E.coli* BL21(DE3) without IPTG induction; 3: pET-katE clones transformed *E.coli* BL21(DE3) with IPTG induction

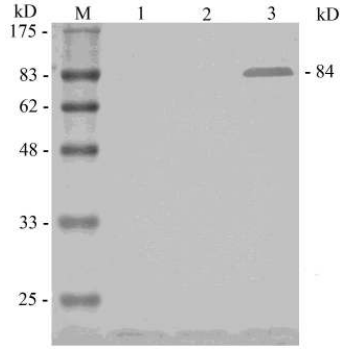


Fig. 9 Western blot analysis of the Kat protein expressed in *E.coli* BL21（DE3） using Ni-NTA conjugate antibody
M: prestained protein marker; 1: BL21(DE3) with IPTG induction; 2: pET-katE clones transformed *E.coli* BL21(DE3) without IPTG induction ; 3: pET-katE clones transformed *E.coli* BL21(DE3) with IPTG induction

The catalase activity of *L.lactis* was also dependent on the concentration of salt. Salt stress

experiments showed that after 8 h incubation, 5·1% of NZ9000 were viable. However, only recombinant *L lactis* exhibited greater salt sensitivity as compared to the parent culture NZ9000 surviving after 8 h. From this result it would appear that this recombinant strain may be involved in responding to salt or osmotic stress. The recombinant *L.lactis* revealed growth difference in different physiological environment. It survived and revived surpassing normal *L.lactis* NZ9000 at low temperature（4□-0□）. It suggests that recombinant *L.lactis* has ability against environment salt stress.

## Sequence analysis

The open reading fram of pET-katE containg kat gene is 2262nt long，and translating protein is 753 amino acids, and its' isoelectric point is 5.67. The nucleotides and amino acids are as followed ( see Fig 10.). The protein sequence was translated as seeing fig 11.

atgtcgcaakataacgaaaagaacccakatcagcaccagtcaccactacacgattccagc… …60
gaagcgaaaccggggatggactcactggcacctgaagacggatctkatcgtccagcggct… ..120
gaaccaacaccgcctggtgcacaacctaccgccccagggagcctgaaagcccctgatacg.....180
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gtgtggtcacgkattcctaagattgacaaaatcccggcgtaa.....2262

Fig.10 Determination of nucleotide sequence of kat katE gene (n1 sequence)

MSQHNEKNPHQHQSPLHDSSEAKPGMDSLAPEDGSHRPAAEPTPPGAQPTAPGSLKAPDT
RNEKLNSLEDVRKGSENYALTTNQGVRIADDQNSLRAGSRGPTLLEDFILREKITHFDHERIPER
IVHARGSAAHGYFQPYKSLSDITKADFLSDTNKITPVFVRFSTVQGGAGSADTVRDIRGFATKF
YTEEGIFDLVGNNTPIFFIQDAHKFPDFVHAVKPEPHWAIPQGQSAHDTFWDYVSLQPETLHNV
MWAMSDRGIPRSYRTMEGFGIHTFRLINAEGKATFVRFHWKPLAGKASLVWDEAQKLTGRDP
DFHRRELWEAIEAGDFPEYELGFQLIPEEDEFKFDFDLLDPTKLIPEELVPVQRVGKMVLNRNP
DNFFAENEQVAFHPGHIVPGLDFTNDPLLQGRLFSYTDTQISRLGGPNFHEIPINRPTCPYHNFQ
RDGMHRMGIDTNPANYEPNSINDNWPRETPPGPKRGGFESYQERVEGNKVRERSPSFGEYYSH
PRLFWLSQTPFEQRHIVDGFSFELSKVVRPYIRERVVDQLAHIDLTLAQAVAKNLGIELTDDQLN
ITPPPDVNGLKKDPSLSLYSIPDGDVKGRVVAILLNDEVRSADLLAILKALKAKGVHAKLLYSR
MGEVTADDGTVLPIAATFAGAPSLTVDAVIVPCGNIADIADNGDANYYLMEAYKHLKPIALAG
DARKFKATIKVADQGEEGIAEADSADGSFMDELLTLMTAHRVWSRIPKIDKIPA.

Fig 11. Sequence of amino acids from KAT protein

It is known that the kat gene is katE because the sequence is homology to katE genes from *Escherichia coli O157:H7 str* and *Escherichia coli str. K-12 substr*, the homology is 98, 99.8 percent, respectively. Analysis of the amino acid sequence of katE of *E.coli* (cloned) revealed that it contains all the typical features of the KAT proteins (Table 2).

Phylogenetic analysis. In order to root a tree of katE from 8 bacteria sequenced to date, a DNA polymerase phylogeny was inferred using the kat genes from those bacteria representing different Phyla which had a high score and the lowest e-values in tblastx searches against bacteria kat gene( Fig.12).

Pair Distances of Untitled ClustalW (Weighted), Percent Similarity in upper triangle in table 2

Percent Identity

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 98.0 | 99.8 | 24.9 | 26.2 | 26.5 | 25.5 | 25.0 | 25.1 | 1 | E.coli katE(cloned).seq |
| | | 97.9 | 24.8 | 26.0 | 26.1 | 25.7 | 25.1 | 25.6 | 2 | E.coli katE Seq #2.seq |
| | | | 24.9 | 26.2 | 26.5 | 25.5 | 25.0 | 25.1 | 3 | E.coliGK12 katE Seq #4.seq |
| | | | | 24.4 | 26.5 | 27.1 | 24.4 | 26.7 | 4 | bacillus subtilis kat E Seq #5.seq |
| | | | | | 26.4 | 25.9 | 58.3 | 27.5 | 5 | hay bacillus cat(ORF) Seq.seq |
| | | | | | | 29.0 | 26.1 | 27.3 | 6 | Pseudomonas katE Seq #8.seq |
| | | | | | | | 24.6 | 27.0 | 7 | Salmonella katE Seq #6.seq |
| | | | | | | | | 26.5 | 8 | Staphylococcus carnosuscat(ORF) Seq.seq |
| | | | | | | | | | 9 | yersinia kat E Seq #7.seq |

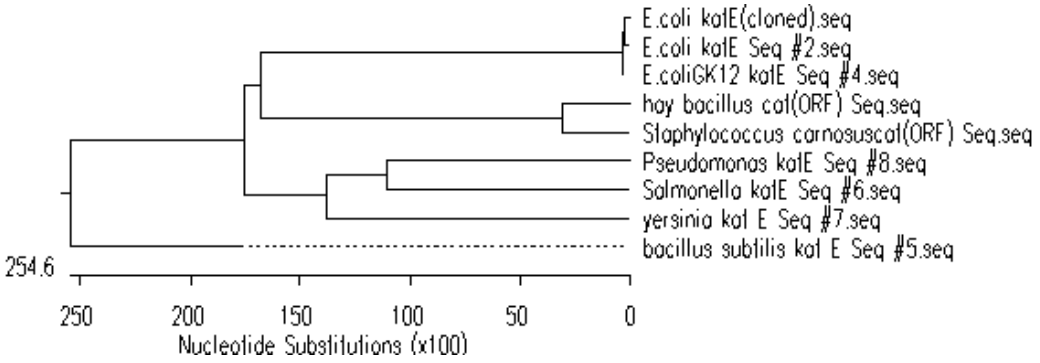
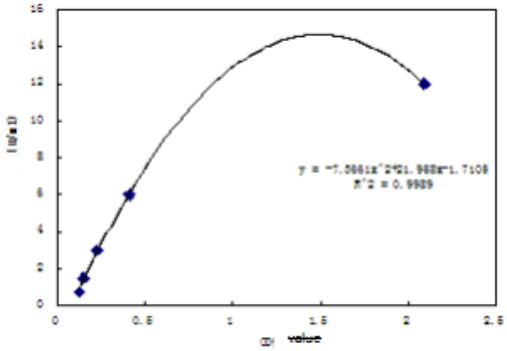



Fig 12. Phylogenetic tree analysis from kat genes of bacteria strains

**Detection of immunization in the blood of experimental animals.** The results were expressed as percentage of blast cells (CD4_, CD8_ T and B cells). ELISA for katE-specific IgG and IgE antibodies was performed by porcine anti-rotavirus A IgG and IgE antibodies as described in the manufacturer's instructions. But the secondary antibody was exchanged by peroxidise-labelled goat anti-porcine IgE (Thermo Fisher Scientific, Bonn, Germany) at a dilution of 1/10 000. The absorbance of each sample was measured at 450 nm.

The concentration of *L.lactis* NZ9000 is $8.4\times10^{8}$cfu/ml, and that of *E.coli* DH5α is $7.2\times10^{6}$cfu/ml.



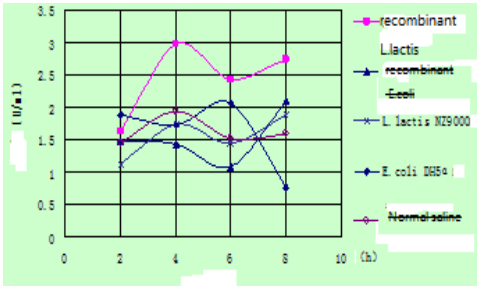
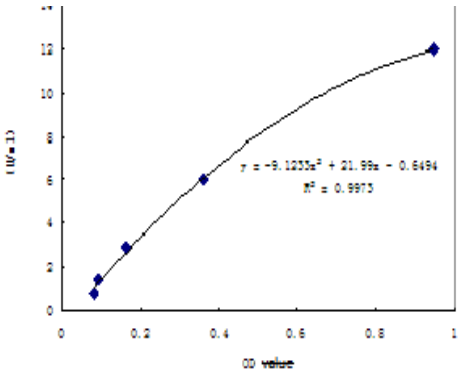



Fig 13. Standard curve of IgG Fig 14. Comparison of IgG among the different treatments



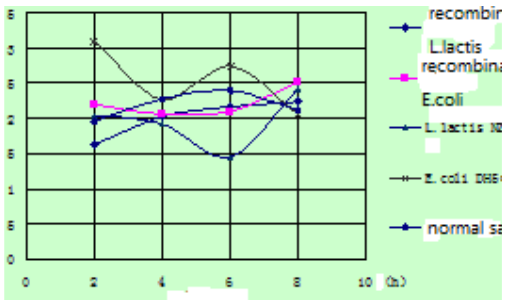



Fig 15. Standard curve of IgE Fig 16. Comparison of IgE among the different treatments

**Comparison of statistical analysis**

The level of IgG from the group with recombinant *L.lactis* NZ9000 treatment was higher than that of other treatments (see Tab 3.) There is a significant difference between them. Variation of Immunoglobulin IgG of the sera from mice with recombinant *L.lactis* NZ9000 was higher than that of other treatments, and difference is significant ($p<0.05$ see Fig 13,14 and Tab3). However, the level of IgE was no difference between them (see Fig15, 16 and Tab4).

Table 3. Comparison of IgG among different treatment groups (u/ml)

| groups | N | 2h | 4h | 6h | 8h |
|---|---|---|---|---|---|
| Recombinant *L.lactis* NZ9000 | 10 | 1.628±0.012 | 2.967±0.008 | 2.424±0.009 | 2.745±0.010 |
| Recombinant *E.coli* DH5α | 10 | 1.483±0.021· | 1.431±0.011· | 1.079±0.013· | 2.010±0.00·9 |
| *L.lactis* NZ9000 | 10 | 1.119±0.011· | 1.745±0.013· | 1.443±0.009· | 1.893±0.008· |
| *E.coli* DH5α | 10 | 1.881±0.010 | 1.732±0.010 | 2.075±0.008 | 0.758±0.009 |
| N.S | 10 | 1.443±0.009· | 1.939±0.011· | 1.516±0.009· | 1.589±0.012· |

Table 4. Comparison of IgE among different treatment groups (u/ml)

| Groups | n | 2h | 4h | 6h | 8h |
|---|---|---|---|---|---|
| Recombinant *L.lactis* NZ9000 | 10 | 1.619±0.006 | 2.30±0.013 | 2.178±0.0010 | 2.245±0.011 |
| Recombinant *E.coli* DH5α | 10 | 2.211±0.010 | 2.061±0.015 | 2.100±0.015 | 2.521±0.010 |
| *L.lactis* NZ9000 | 10 | 2.049±0.011 | 1.917±0.011 | 1.444±0.009 | 2.425±0.011 |
| *E.coli* DH5α | 10 | 3.101±0.009 | 2.283±0.011 | 2.749±0.011 | 2.069±0.012 |
| N.S | 10 | 1.957±0.012 | 2.270±0.009 | 2.405±0.013 | 2.120±0.012 |

The results showed that the concentration of CD4 and CD8 from experimental animals with recombinant *L.lactis* NZ9000 was not higher than that of the other groups. There was no difference between them ($P>0.05$,see Tab 5,6 and Fig 18,20).

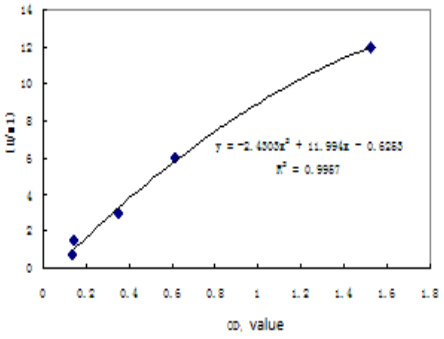



Fig. 17 Standard curve of CD4

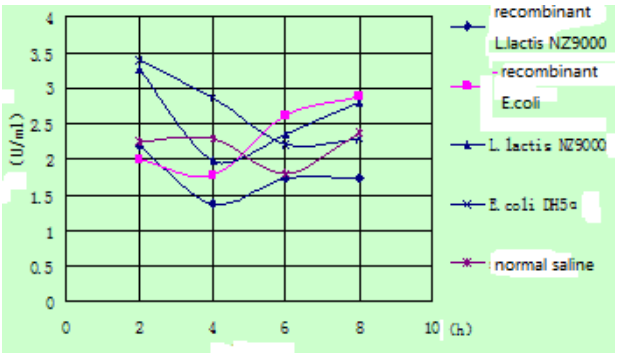



Fig. 18 Variation of CD4 concentration

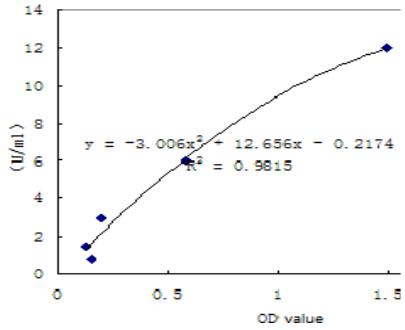



Fig. 19 Standard curve of CD8

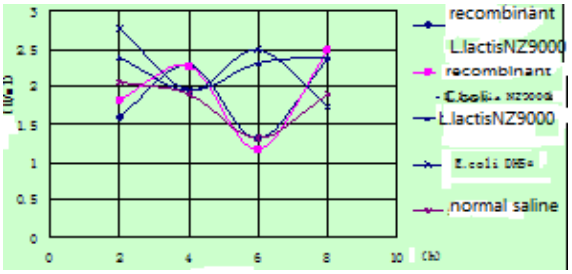



Fig. 20 Variation of CD8 concentration

comparison. And there is no different between them (see Tab 7). The trend of variation showed in figure 21.

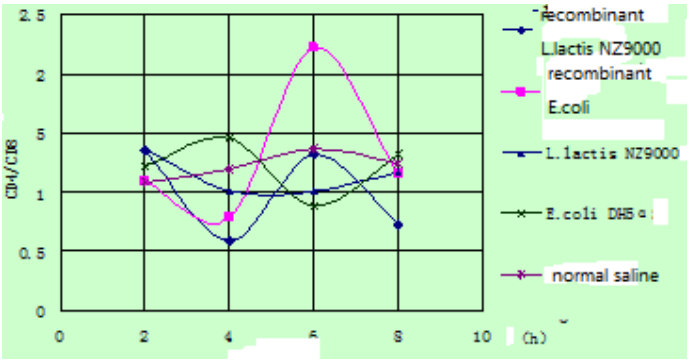



Fig. 21 Variation of CD4/CD8

Tab 5. comparison of CD4 among groups(u/ml)

| Groups | n | 2h | 4h | 6h | 8h |
|---|---|---|---|---|---|
| Recombinant *L.lactis* NZ9000 | 10 | 2.175±0.012 | 1.358±0.012 | 1.739±0.011 | 1.739±0.012 |
| Recombinant *E.coli* | 10 | 2.008±0.009 | 1.783±0.011 | 2.609±0.011 | 2.883±0.014 |

| | | | | | |
|---|---|---|---|---|---|
| *L.lactis* NZ9000 | 10 | 3.2256±0.014 | 1.975±0.013 | 2.350±0.008 | 2.803±0.015 |
| *E.coli* H5α | 10 | 3.378±0.012 | 2.849±0.011 | 2.211±0.013 | 2.289±0.011 |
| *NS* | 10 | 2.240±0.013 | 2.289±0.013 | 1.805±0.012 | 2.375±0.013 |

Tab 6. comparison of CD8 among groups(u/ml)

| Groups | n | 2h | 4h | 6h | 8h |
|---|---|---|---|---|---|
| Recombinant *L.lactis* NZ9000 | 10 | 1.601±0.013 | 2.281±0.010 | 1.317±0.007 | 2.377±0.019 |
| Recombinant *E.coli* | 10 | 1.823±0.011 | 2.272±0.013 | 1.174±0.015 | 2.481±0.018 |
| *L.lactis* NZ9000 | 10 | 2.385±0.016 | 1.945±0.011 | 2.322±0.020 | 2.397±0.016 |
| *E.coli* H5α | 10 | 2.773±0.012 | 1.954±0.013 | 2.483±0.021 | 1.734±0.023 |
| *NS* | 10 | 2.059±0.010 | 1.907±0.012 | 1.317±0.022 | 1.907±0.022 |

Tab 7. comparison ofCD4/ CD8 among groups(u/ml)

| Groups | n | 2h | 4h | 6h | 8h |
|---|---|---|---|---|---|
| Recombinant *L.lactis* NZ9000 | 10 | 1.359±0.075 | 0.595±0.053 | 1.320±0.075 | 0.732±0.091 |
| Recombinant *E.coli* | 10 | 1.044±0.081 | 0.785±0.069 | 2.222±0.083 | 1.162±0.076 |
| *L.lactis* NZ9000 | 10 | 1.369±0.073 | 1.015±0.080 | 1.012±0.081 | 1.70±0.085 |
| *E.coli* H5α | 10 | 1.218±0.036 | 1.458±0.079 | 0.887±0.064 | 1.667±0.078 |
| *NS* | 10 | 1.088±0.054 | 1.200±0.062 | 1.543±0.053 | 1.245±0.081 |

**Survival of the mice**

Five days after the treatment, the mice with *L.lactis* NZ9000 and recombinant *L.lactis* NZ9000 were more actively than that with *E.coli* DH5α.

## Discussion

Report demonstrated the parameter requirement for successful transformation of *L. lactis* NZ9000 strain with electroporation（Gerber, S.D.et al. 2007）. Electroporation transformation was used in the study. A critical factor is that the resistors used in electroporation should be in parallel to shorten the pulse decay time, which should stay between 20 to 40 ms for the best result. The concentration of 2-3% glycine in the media also will provide the optimal growth for the transformation efficiency. Also we investigated the cloning and expression of catalase from *E.coli* in *L.lactis* and the role of oxidation and salt stress in the expression of the catalase of *L.lactis* subsp. The transformants were screened for catalase activity on brain heart infusion (BHI) containing tannic acid (BHI-tannic acid medium) and ampicillin (150 μg/ml), Tannica cid could induce the activity of catalase it is identified in the experiment (**Osawa, R.** 1990.), and this method for cloning catalase gene is feasible.

Many problems were found when genome of *E.coli* was cut with Sau3AI. The intact catalase gene was to be got only by random partly cutting. The fragments molecular weights more than 2000Dt

were insured to be collected. And the restricted enzyme BamHI which could produce the same cohesive 5'ends as Sau3A was used in the test. Many fragments The time should be controlled when the plasmid of pUC18-kat was cut with BamHI because of the sites of BamHI in the kat gene in order to ensure the integrity of gene. In this work, Catalase is induced either by nisin. Moreover, this induction occurs at the appropriate time in anaerobic culture. It may be transcriptional time and results in enhanced catalase expression. The *kat* gene was successfully expressed in the heterologous host *E. coli*.. It was also identified by western-blot. Of the recombinant strain tested, only recombinant *Lactococcus lactis* exhibited greater salt sensitivity compared to the parent culture NZ 9000. This observation suggests that engineering bacteria may be involved in the response of *L. lactis* to salt stress. After 20 min incubation, 70% of the NZ9000 cells remained viable. The effects associated with the catalase insertion were due specifically to changes in recombinant *L.lactis*, strain was exposed to salt, like the other strains. This strain was more resistant to salt than normal strain NZ9000. This may be the result of high catalase levels as a consequence of the high copy number of the plasmid harbouring the catalase gene (Hikmate A, Anette H,2002).

In some cases, catalase genes exhibit sequence homology between the different bacteria strains but they are regulated differently (Schellhorn & Hassan, 1988). It is proved in this study.

The genetic improvement of *Lactococcus lactis* is a matter of biotechnological interest in the food industry and in the pharmaceutical and medical fields. The main metabolism of *L. lactis* is through the anaerobic pathway, fermentation, which produces lactic acid from the available carbohydrates and is used for industrial food production (MartirosyanA.O., et al 2004) .

The report demonstrated that antigen transport would be increased in the absence of the intestinal microflora, it indicates that the gut microflora is an important constituent in the intestines defence barrier. for example production of IgE antibodies, it is upon oral antigen administration.

Abrogation of oral tolerance was due to the absence of intestinal flora. The aberrant IgE response could be corrected by reconstitution of the microflora at the neonatal stage, but not at a later age (Sudo et al. 1997). However, the interest in gastric bacteria has increased only since the isolation of *H. pylori* from the gastric mucosa of human patients with gastroduodenal diseases (Gerber, S.D., et al. 2007,). While immunization with recombinant *L.lactis* containing catalase gene merely induces slightly higher levels of B-cell epitope specific IgG, It is suggest that recombinant *L.lactis* NZ9000 containing catalase gene could stimulate humoral immunity of the animals. Whether contribution, if any, of the T cell response to humoral response leads to qualitatively superior specific IgG antibodies is an interesting consideration to explore in future studies.

CD4+CD8 cells were considered as T regulatory (Treg) cells. Some animals from selected time points were additionally tested for an intracellular antibody against Foxp3.The relative number of CD4 cells was no significantly ($p>0.05$) increased in the probiotic group. No difference was observed between mouse strains in regard to the presence only in the blood of the experimented animals. It is proved that no influence of recombinant *L.lactis* NZ9000 containing catalase genen for cellular immunity of animal could be detected in the blood.

The result in genetic performance of *L. lactis* reveals another industrial advantage for producing new protein and genetic engineering. It is testified by the test of animals survival. Not only it is important in dairy production, it also has potential of use as oral vaccine, foreign protein

production and metabolite through genetic engineering to manipulate *L. lactis* in the researchers' favor.

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