

Expression of recombinant porcine lactoferrin N-lobe in *Pichia methanolica* and its antibacterial activity*

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ABSTRACT

Lactoferrin is a multifunctional, iron-binding glycoprotein found in physiological fluids of mammals. Many functions of lactoferrin are thought to be involved in the N-lobe. In the present study, a gene encoding the N-lobe of porcine lactoferrin was cloned and expressed in *Pichia methanolica*. The gene contains 1038 bp, an “AGG” codon between 1039-1041bp in the PLF gene was replaced with a “TAA” stop codon through primer design and PCR. The sequencing results were aligned with the sequence (Genbank, L77887), indicating that the homology between these two sequences was 99%. The porcine lactoferrin N-lobe (PLfN) was expressed successfully in *Pichia methanolica*, and was secreted into the culture medium. The protein translated from the new ORF contained 346 amino acid residues, including a 17-amino acid signal peptide, its MW was approximately 42 kD, and it possessed antibacterial activity toward *Escherichia coli* ATCC25922. The method established in our study will pave the way for efficient industrial production of recombinant PLfN on a large scale for future utilization of this protein as a feed additive.

KEY WORDS: porcine lactoferrin, N-lobe, recombinant, antibacterial activity, *Pichia methanolica*

INTRODUCTION

Antibiotics have been widely used in the pig industry. Their extensive use and misuse have led, however, to the emergence of drug-resistance (Monroe and Pork, 2000; Hamilton-Miller, 2004) and the presence of antibiotic residues in animal products (Schwarz et al., 2001; Hamilton-Miller, 2004). Therefore, it is urgent to develop a non-antibiotic immunostimulant with high efficiency and low toxicity.

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Lactoferrin (LF), a member of the transferrin family of iron-binding glycoproteins, is an important component of the non-specific immune system, which has been attributed many physiological roles including regulation of iron metabolism (Suzuki et al., 2001; Toshiaki et al., 2006), protection against microbial infection (Pyong et al., 2001), regulation of immune function (Esteban et al., 2005; Chand et al., 2006), stimulation of non-specific immune response (Kamilya et al., 2006), modulation of the inflammatory response (Hayashida et al., 2004). Many of these functions of lactoferrin are, however, thought to involve the N-lobe (van Berkel et al., 1997). This region of lactoferrin did prove to be essential for its antibacterial activity (Haukland et al., 2001; Nakamura et al., 2001; van der Strate et al., 2001). As a consequence, the functions, species specificity and biological roles of the N-lobe of this protein must be further elucidated for the commercial use of lactoferrin.

In the present study, cloning and expression of a gene encoding the N-terminal half (N-lobe) of porcine lactoferrin was performed in *Pichia methanolica*, and the antimicrobial activity of the recombinant porcine lactoferrin N-lobe (PLfN) was examined.

MATERIAL AND METHODS

Materials

Cubes of tissue containing mammary cells were excised from a lactating Yorkshire sow's mammary gland. Trizol reagent, oligo (dT), M-MLV reverse transcriptase and pGEM-3Z vector were purchased from Promega (USA). DNA restriction enzymes and T4 DNA ligase were obtained from Sangon Co. (PRC). PVDF Western blotting membrane and Anti-His 6 × mouse monoclonal antibody were purchased from Roche Diagnostics Limited (Germany). Alkaline phosphatase conjugate goat anti-mouse immunoglobulin (IgG) antibody, alkaline phosphatase, foetal calf serum (FCS), sodium dodecyl sulphate (SDS) polyacrylamide gels, acrylamide, methylene-bisacrylamide, and Coomassie Brilliant Blue R250 were purchased from Promega (USA). Peptone, yeast extract, yeast nitrogen base, and casamino acids were obtained from Difco Laboratory (USA). Prestained molecular weight size marker was purchased from Bio-Rad (USA). *Pichia methanolica* expression kit was purchased from Invitrogen (USA). *E. coli* strain ATCC25922 was provided by Dr. Li from our university.

Growth media and conditions

E. coli DH_{5α} was cultured in LB (Luria-Bertani) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.5). YPAD (Yeast Extract Adenine Peptone

Dextrose) plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, and 0.01% adenine) were used to selected *Pichia methanolica* transformants. MM (1.34% YNB, 4×10^{-5} % biotin, and 2% dextrose) and MD (1.34% YNB, 4×10^{-5} % biotin, and 2% dextrose) plates were used to identify the Mut^s transformants. BMDY (1.0% yeast, 2% peptone, 1.34% YNB, 4×10^{-5} % biotin, 2% dextrose, and 100 mM potassium phosphate) was used to support *Pichia methanolica* growth. For protein expression, yeast were inoculated in BMMY (1.0% yeast, 2% peptone, 1.34% YNB, 4×10^{-5} % biotin, 0.5% methanol, and 100 mM potassium phosphate) medium and grown at 28~30°C.

Cloning of the porcine lactoferrin N-lobe gene

Total RNA was isolated from the mammary gland cells of a 15-day lactating Yorkshire sow using the trizol reagent according to the manufacture's manual. Single-strand cDNA was synthesized by annealing 2 µg of total RNA with 0.5 µg of oligo (dT) in a total volume of 25 µl and using 200 U M-MLV reverse transcriptase at 37°C for 60 min and then 65°C for 10 min. The single-strand cDNA was used as a template for polymerase chain reactions (PCR).

Using the porcine mammary gland cDNA as the template and the synthesized oligonucleotides as primers (Table 1), a fragment encoding the N-lobe portion of

Table 1. Specific primers of porcine lactoferrin N-lobe gene

Gene	Accession number	Primer source	Oligonucleotide sequence	Length (bp)
PLfN	L77887	Pig	5'- CCGGATCCATGAAGCTCTTCATCCCCGC-3' (sense primer, with a <i>Bam</i> HI site)	1038
			5'-GCCAATTCTTACAGGCCCTGGATGGCAGTAAG-3' (antisense primer, with a <i>Eco</i> RI site)	

the porcine lactoferrin gene that contains a 1038 bp coding sequence (cds) of porcine PLfN was amplified by the reverse transcription polymerase chain reaction (RT-PCR) method. The PCR conditions were as follows: reaction mixture containing 1.0 µl of the above template DNA (0.1 µg), 1.0 µl of each primer (20 µM), 5 µl 10 × PCR reaction buffer, 3 µl MgCl₂ (25 µM), 1 µl dNTPs mixed in a final volume of 50 µl. The reaction was run for 30 cycles (denaturing at 94°C for 50 s, annealing at 55°C for 50 s, and extension at 72°C for 2 min). The extension step in the last cycle was run for 10 min. The PCR products were analysed by electrophoresis on 1% agarose gel, purified using a PCR purification kit, and subsequently subcloned into a TA cloning kit pGEM-3Z vector. The recombinant plasmid thus constructed was labeled pGEM-3Z-PLfN. The sequence of the cloned PLfN was confirmed by the dideoxy-chain termination method with a nucleotide sequencer.

Construction of recombinant transfer vector

The PLfN gene was cut from pGEM-3Z-PLfN by digestion with *Bam*HI and *Eco*RI. Using T4 DNA ligase, the PLfN gene was subsequently cloned into multiple cloning sites of the transfer vector pMET-B treated with the same enzymes. The recombinant vector obtained was termed pMET-PLfN.

Transformation of Pichia methanolica and screening of recombinant colonies

Linearized plasmid pMET-PLfN, prepared at 37°C for 3 h in a *Pst*I digestion mixture, was used to transform the PMAD11 or PMAD16 strains of *Pichia methanolica* using an electroporator (MicroPulser, BioRad). The cells were then plated on MD plates and incubated at 28~30°C for 3 to 4 days until colonies came up. MM and MD plates were used to identify the Mut^s which grows normally on MD plates but shows little or no growth on MM plates. A Mut⁺ phenotype suggests that the gene has integrated elsewhere in the chromosomal DNA *via* non-homologous recombination.

Expression of the porcine lactoferrin N-lobe

The cells were cultured overnight in 50 ml of BMDY medium at 28~30°C in a shaking incubator (250 rpm). When OD₆₀₀ reached 2~10 the cells were harvested and inoculated in BMMY to induce expression. The cell cultures were continued and every 24 h for 3 days the culture was supplemented with additional methanol to maintain a concentration of 0.5% methanol. The medium and cell pellets were analysed for protein expression by Coomassie-stained SDS-PAGE and Western blotting.

SDS-PAGE and Western blot analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis were performed to detect the recombinant PLfN in the culture media and cell extracts. SDS-PAGE was performed on a 0.75-mm slab gel (Sambrook et al., 1989). For Western blot analysis, proteins were transferred onto a PVDF membrane. The membrane was subsequently blocked in 1% BSA-TBS (2 h at 4°C), then incubated with a mouse monoclonal antibody (1:2000 diluted in BSA-TBST containing 0.05% Tween 20) for 1 h at room temperature. The membrane was washed, after which alkaline phosphatase-conjugate goat anti-mouse IgG was added. Western-blue-stabilized substrate for alkaline phosphatase was used for detection.

Measurement of antibacterial activity

To evaluate the biological activity of recombinant PLfN expressed in *Pichia methanolica*, agarose diffusion assay and time-kill studies were performed (Wang et al., 2005). The agarose diffusion test was done in standard Petri dishes; the test strain *E. coli ATTC25922* was grown to the stationary phase and mixed with 1% agar. Wells (3.0 mm diameter) were punched in the agar and ten microliters of the purified recombinant PLfN from the culture medium were added. Doxycycline (32 µg/ml) was used as a positive control. After incubation (2-3 d, 37°C) the diameters of inhibition zones were observed. In a time-kill assay, the test strain *Escherichia coli ATTC25922* was grown to the stationary phase, collected by centrifugation and resuspended in MRS medium. Then 0.1 ml of bacterial suspension was added to 4.9 ml of MRS medium to give a final concentration of 10⁶ cfu/ml, and subsequently the purified recombinant PLfN was added. Doxycycline (32 µg/ml) was used as a positive control. Then, the mixtures were incubated anaerobically at 37°C on a shaker (180 rpm). The optical density (OD) was measured at 600 nm in order to evaluate the growth of the bacterium.

Duncan's new multiple-range statistical method was applied to evaluate the significance of differences.

RESULTS

Clone and sequence of the porcine lactoferrin N-lobe gene

In this experiment, we cloned the PLfN gene, which contains 1038 bp (Figure 1). The DNA sequence of the PLfN gene obtained in this work and the deduced amino acid sequence are shown in Figure 2. The sequencing results were aligned with the sequence in Genbank (L77887), showing that the homology between these two sequences was 99%. In this work, an "AGG" codon between 1039-

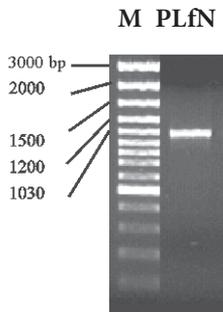


Figure 1. Agar gel electrophoresis of PCR products. M: 3000bp DNA ladder; 1: PLF-N

1041 bp in the porcine lactoferrin gene was replaced with a “TAA” stop codon through primer design and PCR. Therefore, the protein translated from the new ORF contains 346 amino acid residues including a 17-amino acid signal peptide. The molecular weight of the product (346 amino acid residues) is thus expected to be approximately 42 kD.

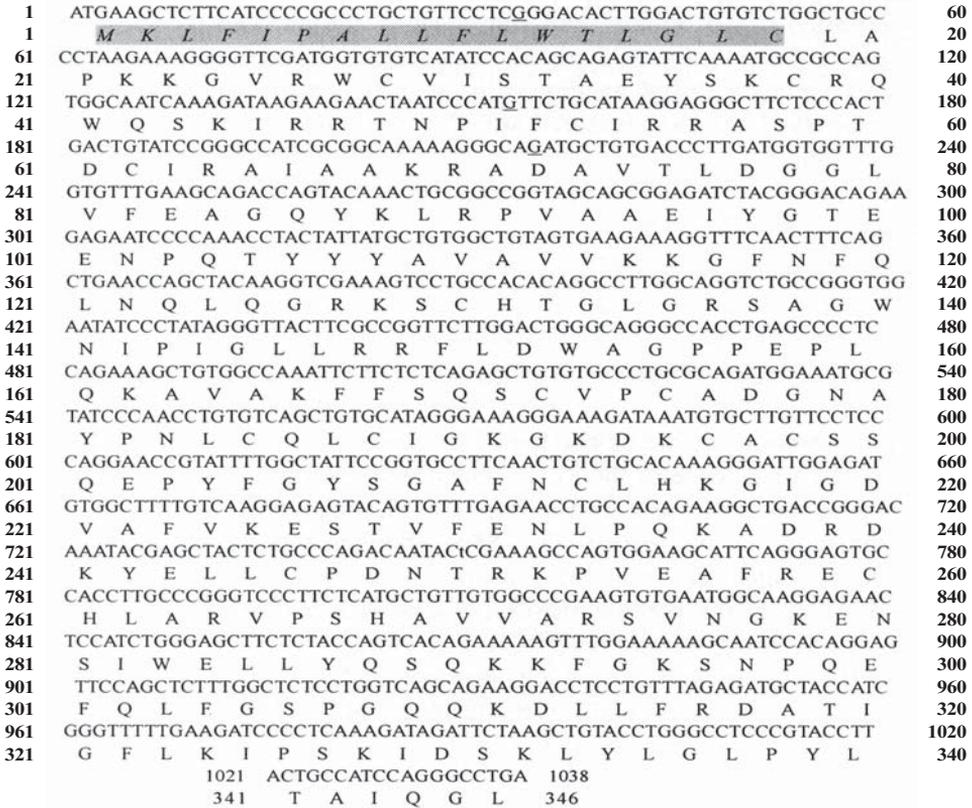


Figure 2. Sequence of the cloned cDNA encoding the PLfN. The cDNA sequence and the deduced amino acid sequence were shown. The points mutation were underlined and the 17-amino acid signal peptide was writhed in italics

Expression of the recombinant porcine lactoferrin N-lobe in Pichia methanolica

To confirm expression of the recombinant PLfN, the recombinant protein was expressed in the BMMY culture for 3 days and every 24 h the culture medium and cells were collected separately by low-speed centrifugation. The culture supernatant and cell lysates were examined by SDS-PAGE and Western blot analysis. As shown in the Figure 3, a clear band of approximately 42 kD, which corresponds to the

molecular weight of recombinant PLfN, was observed in the sample (Figure 3A), implying that recombinant PLfN is well expressed. Furthermore, the expression was well proved by Western blotting analysis (Figure 3B).

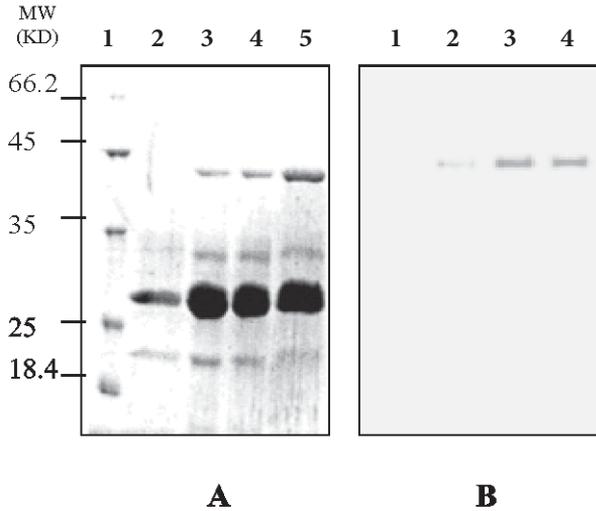


Figure 3. Detection of the recombinant PLfN secreted into the culture medium. Following three days of growth after inducement, culture samples were harvested, centrifuged to separate the culture media and the cell pellet. A. SDS-PAGE analysis of recombinant PLfN from the culture media samples of transformants. The culture medium was dissolved by SDS-PAGE on 12% gel and subsequently visualized by staining with Coomassie blue. Lane 1, molecular marker; Lane 2, negative control the culture medium of host *Pichia methanolica* with vector only; lane 3, induced 24 h; lane 4, induced 48 h; lane 5, induced 72 h. B. Western blot analysis of recombinant PLfN from the culture media samples of transformants. Lane 1, negative control using host *Pichia methanolica* with vector only; lane 2, induced 24 h; lane 3, induced 48 h; lane 4, induced 72 h

Antibacterial activity of the recombinant porcine lactoferrin N-lobe

The agarose diffusion assay showed that the recombinant PLfN from the culture could inhibit the growth of *Escherichia coli* ATTC 25922 effectively (Figure 4), whereas the control had no antibacterial activity.

To further evaluate the antibacterial activity of recombinant PLfN from the culture, time-kill studies were conducted. In this experiment, bovine lactoferrin and doxycycline were used as positive controls. The results from Figure 5 show that the expression culture exhibited bactericidal activity compared with the control culture. The results indicate that the difference between the recombinant PLfN and the control was significant, indicating successful expression of recombinant PLfN in *Pichia methanolica* with antibacterial bioactivity.

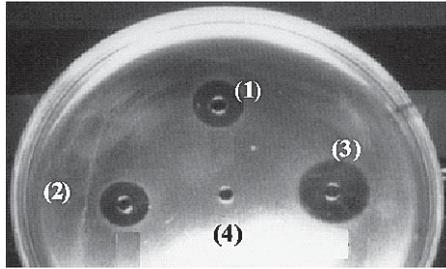


Figure 4. Inhibiting bacterial growth by recombinant PLfN expressed in *Pichia methanolica* on *Escherichia coli* ATCC25922. The analysis method is described in the Material and methods. (1), (2) Treated with ten microliters of recombinant PLfN; (3) Treated with doxycycline (32 $\mu\text{g}/\text{ml}$); (4) The control, treated with supernatant of normal culture of *Pichia methanolica* (with vector only)

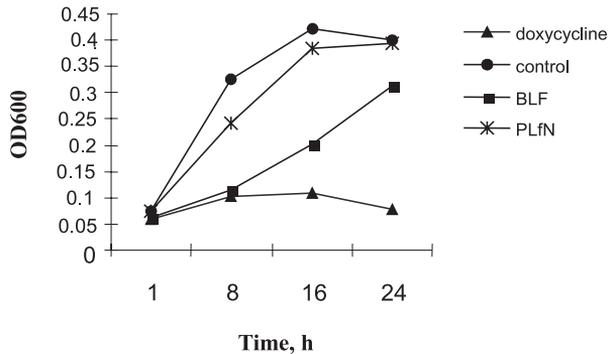


Figure 5. Bioactivity analysis of recombinant PLfN obtained from *Pichia methanolica* (transformants). Recombinant PLfN was added into the bacterial suspension and was then incubated on a shaker anaerobically (180 rpm) at 37°C. The OD was measured at 600 nm to evaluate the growth of the bacterium (*Escherichia coli* ATCC25922). Bovine lactoferrin (BLF, 32 $\mu\text{g}/\text{ml}$) and doxycycline (32 $\mu\text{g}/\text{ml}$) were used as a positive control. The control was treated with supernatant of normal culture of *Pichia methanolica* (with vector only)

DISCUSSION

The major objective of our work was to produce the porcine lactoferrin N-lobe on a large scale with minimum expenditure and to study the significance and species specificity of the N-lobe of lactoferrin. It was demonstrated that the N-terminal region of lactoferrin is important to the antibacterial activity of lactoferrin (Kang et al., 1996; Kimura et al., 2000). Due to the susceptibility of the lactoferrin N-lobe to proteolytic enzymes (Shimazaki et al., 1993), it is difficult to separate the N-lobe from native lactoferrin. Expression systems other than yeast have been utilized to produce PLfN. Besides the expression of the human lactoferrin N-lobe in baby hamster kidney cells (Day et al., 1993) and bovine lactoferrin N-lobe in insect cells

(Nakamura et al., 2001), there are no reports on porcine lactoferrin N-lobe expression using *Pichia methanolica*. The *Pichia methanolica* expression system is now being used with increasing frequency. Some exogenous proteins have been expressed thus far (Choi et al., 2002), and certain advantages including fermentation methods and efficient promoters are well documented. In the present study, the expression of the PLfN in *Pichia methanolica* and its antibacterial activity were observed. It was shown that the recombinant PLfN expressed in *Pichia methanolica* has biological activity and can inhibit the growth of *E. coli* ATTC25922 effectively. Furthermore, the levels of the recombinant PLfN are sufficient for structural, functional and species specificity studies on the N-lobe of lactoferrin.

It is well known that during weaning, piglets encounter severe stressors that result in reduced growth (Odle, 1996), precipitating the onset of postweaning diarrhoea, bacterial overgrowth and villus atrophy syndrome (Hampson, 1986). Maternal milk not only contains easily digestible nutrients, but also bioactive factors (Hamosh, 1998; Xu, 1998). Lactoferrin is one of the most important bioactive proteins in milk, and most of the functions of lactoferrin are involved in the N-lobe. So in the present study, we used *Pichia methanolica* as a host to express and secrete recombinant PLfN through fermentation methods; utilizing *Pichia methanolica* directly as a feed additive can be considered. We hope that the method established in our study will pave the way for efficient industrial production of recombinant PLfN on a large scale for its further utilization in the near future.

CONCLUSIONS

In this study, we have succeeded in expressing and producing recombinant PLfN in *Pichia methanolica*, and the recombinant PLfN has bactericidal bioactivity. Eventually, recombinant PLfN may serve as an additive for animals as an aid in the modulation of certain physiological functions of the intestinal system. We hope to apply it in pig feeds to overcome weaning stress problems in the future.

REFERENCES

- Chand R.K., Sahoo P.K., Kumari Jaya, Pillai Bindu R., Mishra B.K., 2006. Dietary administration of bovine lactoferrin influences the immune ability of the giant freshwater prawn *Macrobrachium rosenbergii* (de Man) and its resistance against *Aeromonas hydrophila* infection and nitrite stress. *Fish Shellfish Immunol.* 21, 119-129
- Choi J., Kim E.E., Park Y.I., Han Y.S., 2002. Expression of the active human and duck hepatitis B virus polymerases in heterologous system of *Pichia methanolica*. *Antivir. Res.* 55, 279-290
- Day C.L., Anderson B.F., Tweedie J.W., Baker E.N., 1993. Structure of the recombinant N-terminal lobe of human lactoferrin at 2.0 Å resolution. *J. Mol. Biol.* 232, 1084-1100

- Esteban M.A., Rodriguez A., Cuesta A., Meseguer J., 2005. Effects of lactoferrin on non-specific immune responses of gilthead seabream (*Sparus auratus* L.). *Fish Shellfish Immunol.* 18, 109-204
- Hamilton-Miller M.T., 2004. Antibiotic resistance from two perspectives: man and microbe. *Int. J. Antimicrobial Agents* 23, 209-212
- Hamosh M., 1998. Protective function of proteins and lipids in human milk. *Biol. Neonate* 74, 163-176
- Hampson D.J., 1986. Alternations in piglet small intestinal structure at weaning. *Res. Vet. Sci.* 40, 32-40
- Haukland H.H., Ulvatne H., Aandvik K., Vorland L.H., 2001. The antimicrobial peptides lactoferricin B and magainin 2 cross over the bacterial cytoplasmic membrane and reside in the cytoplasm. *FEBS Lett.* 508, 389-393
- Hayashida K., Kaneko T., Takeuchi T., Shimizu H., Ando K., Harada E., 2004. Oral administration of lactoferrin inhibits inflammation and nociception in rat adjuvant-induced arthritis. *J. Vet. Med. Sci.* 66, 149-154
- Kamilya D., Ghosh D., Bandyopadhyay S., Mal B.C., Maiti T.K., 2006. In vitro effects of bovine lactoferrin, mushroom glucan and *Abrus* agglutinin on Indian major carp, catla (*Catla catla*) head kidney leukocytes. *Aquaculture* 253, 130-139
- Kang J.H., Lee M.K., Kim K.L., Hahn K.S., 1996. Structure–biological activity relationships of 11-residue highly basic peptide segment of bovine lactoferrin. *Int. J. Pep. Protein Res.* 48, 357-363
- Kimura M., Nam M.S., Ohkouchi Y., Kumura H., Shimazaki T.K., Yu D.Y., 2000. Antimicrobial peptide of Korean native goat lactoferrin and identification of the part essential for this activity. *Biochem. Biophys. Res. Commun.* 268, 333-336
- Monroe S., Polk R., 2000. Antimicrobial use and bacterial resistance. *Curr. Opin. Microbiol.* 3, 496-501
- Nakamura I., Watanabe A., Tsunemitsu H., Lee N.Y., Kumura H., Shimazaki K.I., Yagi Y., 2001. Production of recombinant bovine lactoferrin N-lobe in insect cells and its antimicrobial activity. *Protein Express. Purif.* 21, 424-431
- Odle J., Zijlstra T., Donovan S.M., 1996. Intestinal effects of milkborne growth factors in neonates of agricultural importance. *J. Anim. Sci.* 74, 2509-2522
- Pyeong W.P., Gerald B.P., Michael T.H., Bernfield M., 2001. Exploitation of syndecan-1 shedding by *Pseudomonas aeruginosa* enhances virulence. *Nature* 411, 98-102
- Sambrook J., Fritsch E.F., Maniatis T., 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Schwarz S., Kehrenberg C., Walsh T.R., 2001. Use of antimicrobial agents in veterinary medicine and food animal production. *Int. J. Antimicrobial Agents* 17, 431-437
- Shimazaki K., Tanaka T., Kon H., Oota K., Kawaguchi A., Maki Y., Sato T., 1993. Separation and characterization of the C-terminal half molecule of bovine lactoferrin. *J. Dairy Sci.* 76, 946-955
- Suzuki Y.A., Shin K., Lonnerdal B., 2001. Molecular cloning and functional expression of a human intestinal lactoferrin receptor. *Biochemistry* 40, 15771-15779
- Uchida T., Oda T., Sato K., Kawakami H., 2006. Availability of lactoferrin as a natural solubilizer of iron for food products. *Int. Dairy J.* 16, 95-101
- Van Berkel P.H., Geerts M.E., van Veen H.A., Mericskay M., de Boer H.A., Nuijens J.H., 1997. N-terminal stretch Arg2, Arg3, Arg4 and Arg5 of human lactoferrin is essential for binading to heparin, bacterial lipopolysaccharide, human lysozyme and DNA. *Biochem. J.* 328, 145-151
- van der Strate B.W., Beljaars L., Molema G., Harmsen M.C., Meijer D.K., 2001. Antiviral activities of lactoferrin. *Antivir. Res.* 52, 225-239
- Wang Y.Z., Wu X.F., Liu G.F., Cao C.P., Huang H.Q., Xu Z.R., Liu J.X., 2005. Expression of porcine lactoferrin by using recombinant baculovirus in silkworm, *Bombyx mori* L., and its purification and characterization. *Appl. Microbiol. Biotechnol.* 69, 385-389
- Xu R.J., 1998. Bioactive peptides in milk and their biological and health implications. *Food Rev. Int.* 14, 1-16