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Sequence comparison of the lactoferrin of various animal species and its prospects as an animal feed additive

Hamong Suharsono [1], Ni Luh Wayan Yulia Mirayanti [2], Nengah Kerta Besung [3], Ni Putu Sutrisna Dewi [4, 5], Ni Putu Novi Aritayanti [4], Made Sumitha Kameswari [4], Bayu Krisna Mahardika [5], Ida Bagus Kade Suardana [6], Xiaole Qi [7] and Gusti Ngurah Mahardika [5, 6, *]

[1] *Biochemistry Laboratory, Faculty of Veterinary Medicine Udayana University of Bali, Jl. PB Sudirman, Denpasar 80225, Indonesia.*
[2] *Post Graduate School of Biology, Faculty of Mathematics and Natural Sciences, Udayana University of Bali, Kampus Bukit Jimbaran, Badung, Bali, Indonesia.*
[3] *Microbiology Laboratory, Faculty of Veterinary Medicine Udayana University of Bali, Jl. PB Sudirman, Denpasar 80225, Indonesia.*
[4] *Post Graduate School of Veterinary Medicine, Faculty of Veterinary Medicine Udayana University of Bali, Jl. PB Sudirman, Denpasar 80225, Indonesia.*
[5] *The Animal Biomedical and Molecular Biology Laboratory, Udayana University of Bali, Jl. Sesetan-Markisa 6, Denpasar 80226, Indonesia.*
[6] *Virology Laboratory, Faculty of Veterinary Medicine Udayana University of Bali, Jl. PB Sudirman, Denpasar 80225, Indonesia.*
[7] *Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, State Key Laboratory of Veterinary Biotechnology, 678 Haping Road, Xiangfang District, Harbin, 150069, P.R. China.*

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Abstract

Lactoferrin as a feed additive is worth to be explored. However, reports on its application are contradictory, which might be associated with the genetic relatedness. Here we compare the lactoferrin nucleotide and its deduced amino acid sequence. Sequence data for lactoferrin of various animal and human were download from GenBank. The phylogenetic relatedness was inferred and the amino acid sequences were aligned to identify conserved and polymorphic sites. The protein three-dimension structures were estimated using online software. The result showed the lengths of lactoferrin, lactotransferrin or ovotransferrin are 703–711 residues. The phylogeny showed that the lactoferrins of buffalo, cow, goat, pig, camel, and horse formed one group; monkey, human, and gorilla formed a second group; and dog and cat formed a third group. Chicken ovotransferrin was an outgroup. Genetic distances between groups were 0.242–0.061, while smallest span between taxa was 0.016 (human to gorilla) and the highest was 0.612 (chicken to goat). The conserved residues spanned from the amino terminus to the carboxy terminus. There are 27 conserved cysteine residues. N-link glycosylation of the "NXS" and "NXT" motives of lactoferrin diverge between species. A species specific or group specific lactoferrin supplement should be beneficial to animal production.

Keywords: Lactoferrin; Ovotransferrin; Feed additive; Phylogeny; Polymorphic sites; N-link glycosylation motives; Modelling

* Corresponding author: Gusti Ngurah Mahardika; Email: gnmahardika@unud.ac.id

1 Introduction

Feed additive is a key to the successful development of animal origin food production. As per the Food Agriculture Organization (FAO) official definition, a feed additive is "any intentionally added ingredient not normally consumed as feed by itself, whether or not it has nutritional value, that affects the characteristics of feed or animal products (micro-organisms, enzymes, acidity regulators, trace elements, vitamins and other products fall within the scope of this definition depending on the purpose of use and method of administration)" (www.fao.org) .

Feed additives are of particularly high interest for newborn animals because they ensure the survival rate and growth performance of the animal in the production phase. The exploration of feed additives is extraordinarily important in the era of antibiotic restriction. Antibiotics are also key for the advancement of animal production. Antibiotics have been used to treat and prevent disease, increase feed conversion, and preserve food (Kirchhelle, 2018) . However, there have been increasing conflicts regarding its application as growth promotor because it is believed that the unregulated use of antibiotics in agriculture contributes to antimicrobial resistance (AMR) (Hao et al., 2014; Kirchhelle, 2018). Antimicrobial resistance become even an emerging foodborne pathogen (Koluman and Dikici, 2013) . The amount of antibiotics in use is indeed alarming. The global averages of annual consumption of antimicrobials can reach 170 mg/kg body weight of cattle, chickens, and pigs (Van Boeckel et al., 2015). Interventions that restrict antibiotic use in food-producing animals are associated with a reduction in the presence of antibiotic-resistant bacteria in these animals. A smaller body of evidence suggests a similar association in the studied human populations, particularly those with direct exposure to food-producing animals. The calculated absolute risk reduction of the prevalence of AMR in animals with interventions is up to 15% (Tang et al., 2017).

Lactoferrin as a feed additive is worth exploring to increase survival and growth rate, especially in young animals, and to reduce bacterial infection. Lactoferrin (LF), also known as lactotransferrin (LTF), is an iron transport or binding highly glycosylated protein (Garcia-Montoya et al., 2012; Karav et al., 2017; Satue-Gracia et al., 2000), which provides anti-oxidant, anti-bacterial and other protective effects, and presents in various secretory fluids, such as milk, saliva, tears, nasal secretions, and in non-specific polymorphonuclear immune cells. Human colostrum and milk have the highest concentration of lactoferrin, reaching as high as 8 mg/ml (Garcia-Montoya et al., 2012; Sanchez et al., 1992). Recombinant lactoferrin has been added and marketed in human infant formula to enrich bovine milk (Satue-Gracia et al., 2000).

The advantageous effect of the use of lactoferrin in animal feed has not yet been heavily much explored. The most prominent effect of lactoferrin is that it bridges innate and adaptive immune function in mammals. Its protective effects range from anticancer, anti-inflammatory and immune modulator activities to antimicrobial activities against a large number of microorganisms and viruses (Garcia-Montoya et al., 2012; Giansanti et al., 2005; Sakai et al., 2005; Teraguchi et al., 2004; Tomita et al., 1991; van der Strate et al., 2001).

Lactoferrin feed addition should improve animal production performance. Compared with human milk, the concentration of this protein in animal milk is very low. Bovine milk contains less than 0.2 mg/ml, while pig and horse milk contain no more than 2 mg/ml lactoferrin (Masson and Heremans, 1971). However, there is still a small body of research exploring the effect of lactoferrin as a feed additive in animal performance, and the results are contradictory. Positive effects have been shown in some agricultural animals such as neonatal calves (Joslin et al., 2002; Prenner et al., 2007; Robblee et al., 2003; Schottstedt et al., 2005), piglets (Hu et al., 2012; Jahan et al., 2017; Lee et al., 2010; Pagheh et al., 2018; Shan et al., 2007; Tang et al., 2009; Wang et al., 2007; Wang et al., 2006; Wang et al., 2008), fish (Chand et al., 2006; Khuyen et al., 2017; Kumari et al., 2003; Ulloa et al., 2016), and very occasionally on poultry (Hung et al., 2010; Jean et al., 2016). However, little to no promising effects have been reported too (Connelly and Erickson, 2016; Cowles et al., 2006; English et al., 2007; Geier et al., 2011; Henry and Alexis, 2009; Shea et al., 2009).

Here we conducted simple bioinformatic review to compare the mRNA sequence lactoferrin from various animals and humans with its deduced amino acid sequences to gain new insights into applying lactoferrin as a feed supplement in animals.

2 Material and methods

cDNA sequence data for lactoferrin, lactotransferrin or ovotransferrin of various species and human were downloaded from GenBank. The sequences were trimmed before the start codon and after the stop codon of open reading frames annotated in the GenBank. The sequence accession numbers are listed in Table 1. The sequences were aligned using ClustalW, available in the Mega-X package (Kumar et al., 2018). The evolutionary history was inferred using the

neighbor-Joining method (Saitou and Nei, 1987) with a bootstrap test of 1000 replicates (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). Evolutionary analyses were conducted in MEGA-X (Kumar et al., 2018). Protein modeling of the lactoferrin of selected species was performed with the online resource PYRE2 (http://www.sbg.bio.ic.ac.uk) (Kelley et al., 2015). Protein models were visualized with RasWin 2.7.5.2 (www.rasmol.org). N-link glycosylation motives of "NXS" or "NXT" (Chuang et al., 2012) were surveyed using MEGA-X.

3 Results

The accession number, protein name, sequence origin, and protein length for each species are listed in Table 1. The source of the sequences was mRNA sequencing and was predicted from genomic sequences. Given protein names were lactoferrin, lactotransferrin, and ovotransferrin, conalbumin or ovalbumin. The lengths were 703–711 residues. Some sequences were annotated as RefSeq in the database.

Table 1 Accession number of lactoferrin of various animal species and human

No.	Common Name	Species origin	Protein name	Origin sequence	Accession Number	Amino acid sequence length	References
1	Human	*Homo sapiens*	Lactoferrin	Prostate cDNA sequencing	M93150	711	Unpublished
2	Monkey	*Macaca cyclopis*	Lactoferrin	mRNA cloning	EU523857	710	Unpublished
3	Goat	*Capra hircus*	Lactoferrin	mRNA sequencing	U53857	708	Unpublished
4	Pig	*Sus scrofa*	Lactoferrin	mRNA sequencing mammary gland	M81327	703	Unpublished
5	Buffalo	*Bubalus bubalis*	Lactoferrin	mRNA sequencing	AJ005203	708	(Karthikeyan et al., 1999; Karthikeyan et al., 2000)
6	Horse	*Equus caballus*	Lactotrans-ferrin	mRNA sequencing	NM_001163974 (RefSeq)	708	(Hestand et al., 2015; Kolm et al., 2007; Pearl and Roser, 2014; Sharma et al., 1999)
7	Camel	*Camelus dromedarius*	Lactoferrin	mRNA mammary gland	AJ131674	708	(Kappeler et al., 1999)
8	Cattle	*Bos taurus*	Lactoferrin	mRNA lactating mammary gland	L19981	708	(Seyfert et al., 1994)
9	Dog	*Canis lupus familiaris*	Lactotransferrin	mRNA sequencing	XM_541903 (RefSeq)	708	(Berlov et al., 2007; Kida et al., 2006; Morinha et al., 2012)
10	Cat	*Felis catus*	Lactotransferrin	Predicted from genomic sequence	XM011291127 (RefSeq)	708	Unpublished
11	Gorilla	*Gorilla gorilla*	Lactoferrin	mRNA fibroblast	KT006755	711	(Barber et al., 2016)

12	Chicken	*Gallus gallus*	transferrin (ovotransferrin/ conalbumin / ovalbumin)	mRNA sequencing	NM_205304 (RefSeq)	705	(Butterworth et al., 1975; Cochet et al., 1979; Dorland et al., 1979; Labas et al., 2015; Palmiter et al., 1978; Rabbani et al., 2011; Shen et al., 2010; Thibodeau et al., 1978; Watanabe et al., 2008)

The inferred phylogeny of the lactoferrin cDNA of various species and human is presented in Figure 1. The phylogeny shows that the sequences of the lactoferrin of buffalo, cow, goat, pig, camel, and horse form separate clusters from the lactoferrin of monkey, human, and gorilla. For discussion purposes, we clustered the lactoferrin of buffalo, cow, goat, pig, camel, and horse into Group A, monkey, human, and gorilla into Group B, and dog and cat into group C. Chicken ovotransferrin was an outgroup in our analysis. Genetic distances between species under study are listed in Table 2. The overall genetic distance was 0.279. Average genetic distances within Group A, B, and C were 0.182, 0.056, and 0.141, respectively. Genetic distances between groups A and B, A and C, A and D, B and C, B and D, and C and D were 0.250, 0.242, 0.591, 0.244, 0.601, and 0.579, respectively.

Table 2 Estimates of Evolutionary Divergence between lactoferrin ORF sequences of human and various animal species

	Buffalo	**Camel**	**Cat**	**Cattle**	**Chicken**	**Dog**	**Goat**	**Gorilla**	**Horse**	**Human**	**Monkey**
Buffalo											
Camel	0.197										
Cat	0.262	0.208									
Cattle	0.037	0.208	0.263								
Chicken	0.598	0.582	0.588	0.589							
Dog	0.264	0.208	0.141	0.274	0.569						
Goat	0.057	0.199	0.258	0.056	0.612	0.264					
Gorilla	0.266	0.217	0.249	0.27	0.605	0.23	0.27				
Horse	0.233	0.169	0.208	0.237	0.572	0.191	0.236	0.206			
Human	0.269	0.217	0.25	0.272	0.602	0.233	0.271	0.016	0.211		
Monkey	0.268	0.231	0.258	0.272	0.597	0.246	0.273	0.078	0.219	0.073	
Pig	0.227	0.188	0.254	0.231	0.595	0.253	0.227	0.261	0.224	0.261	0.247

The taxa were ordered alphabetically. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA-X (Kumar et al., 2018).

Polymorphic and conserved amino acid residues of the lactoferrin of various animals and human as well as the N-link glycosylation of the "NXS" and "NXT" motives are shown in Figure 2. The polymorphic residues are uncolored and the conserved resides are orange for cysteine and green for residues other than cysteine. The N-link glycosylation of the "NXS" and "NXT" sites are marked red and blue, respectively, in Figure 2. The figure shows there are polymorphic and conserved residues spanning the whole protein. There are 27 conserved cysteine residues along the amino acid sequence (orange in Figure 2). The N-link glycosylation of "NXS" and "NXT" motives between species diverge. Protein modeling of the lactoferrin of cow, human, and chicken is presented in Figure 3. All lactoferrins pose two globular domains. The secondary structures are different in various parts of the protein.

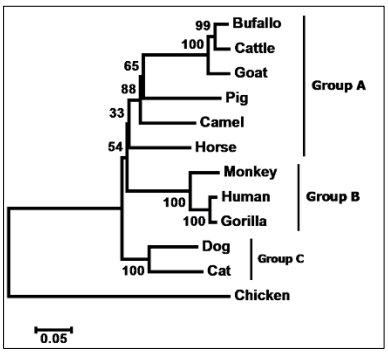

Figure 1 Evolutionary relationships of lactoferrin open reading frame of human and various animal species. The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987) The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA-X (Kumar et al., 2018)

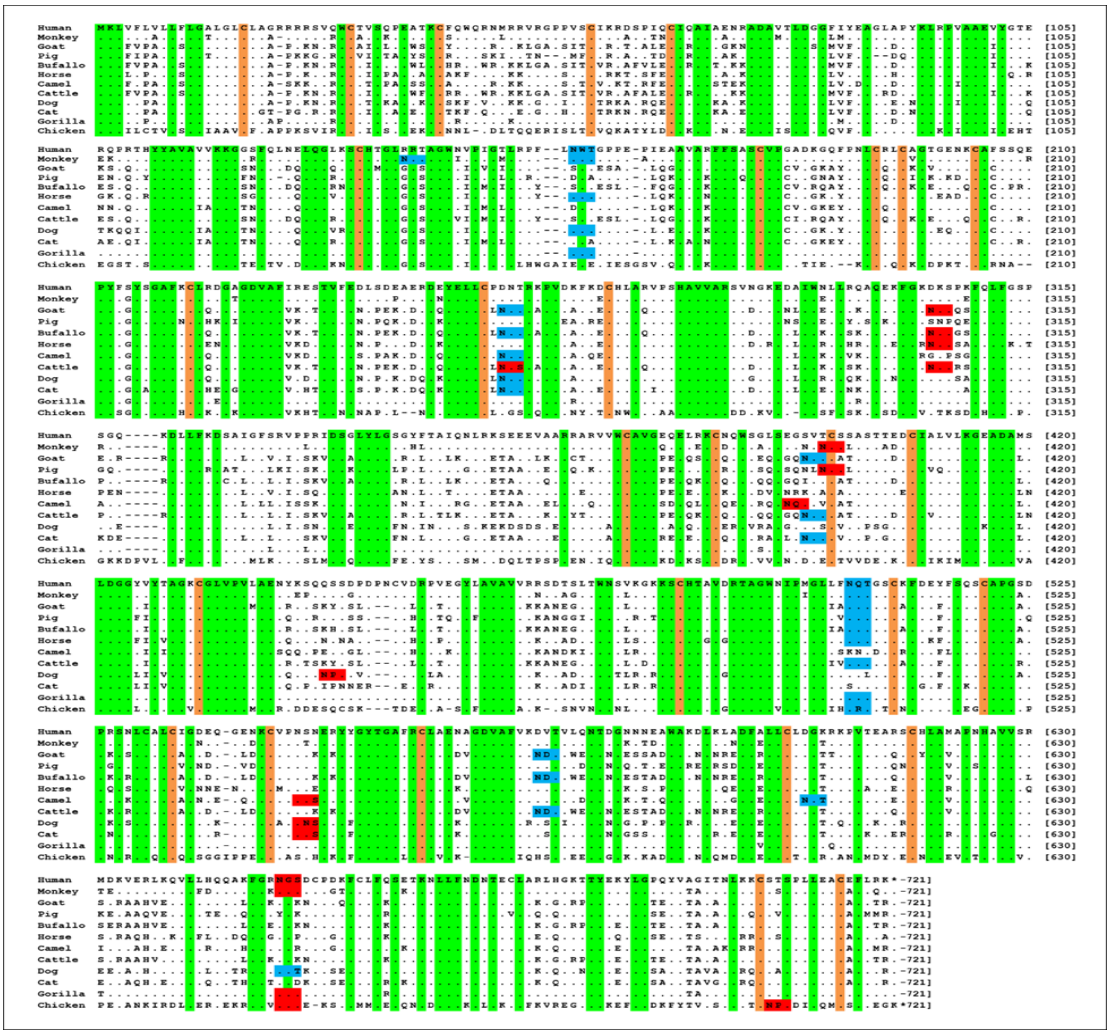

Figure 2 Polymorphic and conserved amino acids of lactoferrin of various animals and human. The polymorphic residues are uncolored, while the conserved residues are colored orange for cysteine and green for residues other than cysteine. N-link glycosylation sites of “NXS” motive are marked red, while “NXT” motives are blue

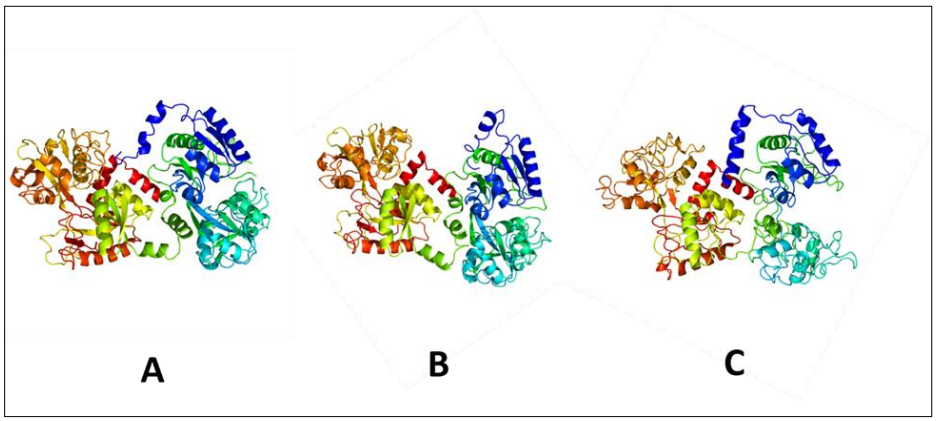

Figure 3 Cartoon peptide modeling of bovine (A), human (B), and chicken (C). Images are colored by inverted rainbow from N- to C-terminus. Protein modeling was performed with the online resource PYRE2 (http://www.sbg.bio.ic.ac.uk) (Kelley et al., 2015). Protein models were visualized with RasWin 2.7.5.2 (www.rasmol.org)

4 Discussion

A previous phylogenetic study of the transferrin family showed that this protein has been diversified into distinct subfamilies, including serotransferrin, ovotransferrin, lactoferrin, melanotransferrin, the inhibitor of carbonic anhydrase, pacifastin, and the major yolk protein in sea urchin (Mohd-Padil et al., 2013). Considering that one main function of this protein is anti-infection, it is believed that genetic conflicts between microbes and their hosts are an important source of the evolutionary innovation of lactoferrin (Barber et al., 2016). The pattern of phylogeny found in our analysis has also been published previously (Akumbugu and Olusegun, 2017). We believe that the sequences of the lactoferrin ORFs analyzed in this study represent respective species, although minimum nucleotide and nonsynonymous amino acid variations do exist between breeds, as previously shown in Indian goat breeds (Anjusekar et al., 2018).

Lactoferrin and transferrin have similar amino acid compositions, secondary and tertiary structures (Bluard-Deconinck et al., 1974; Querinjean et al., 1971). Our observation adds new information, that lactoferrin is a cysteine rich protein with 27 conserved cysteine residues. This is a unique pattern that needs further investigation. Cysteine-rich mini-proteins in humans have been frequently described as ligands for G protein- and enzyme-coupled receptors, transporters, extracellular enzyme inhibitors, and antimicrobial peptides (Lavergne et al., 2012). The information regarding cysteine richness in large proteins is limited. The pattern of the cysteine framework in lactoferrin also needs further investigation because it was found to be related to vital biological functions (Lavergne et al., 2012). Three members of the cysteine-rich protein (CRP) family, namely CRP1, 2, and 3, have been implicated in the processes of cell proliferation and differentiation (Louis et al., 1997).

Protein modeling shows that all lactoferrins have two lobes: an amino-lobe (N-lobe) and a carboxy-lobe (C-lobe), as previously described (Anderson et al., 1987; Gomme et al., 2005). Each lobe forms a deep cleft as a binding site of iron ions (MacGillivray et al., 1998; Mason et al., 1996). In addition to iron, lactoferrin is able to bind, copper, zinc and manganese ions, as well as lipopolysaccharides (LPS), lipoteichoic acid, heparan sulfate (HS), DNA and RNA (Baker et al., 2003; Bennett and Davis, 1982; Ellison et al., 1988; Legrand et al., 2004; Leitch and Willcox, 1999; Rodriguez-Franco et al., 2005; van der Strate et al., 2001). An in-silico comparison report showed that the iron binding site, DNA and RNA binding sites, signal peptides and transferrin motifs were highly conserved between various species (Sohrabi et al., 2014).

Our phylogeny shows that human and some animal lactoferrins form four distinct groups, in which chicken ovotransferrin is an outgroup. This concords with a previous finding. A previous phylogenetic analysis that excluded dog, cat, and chicken lactoferrins showed that these proteins are divided into two distinct groups. One represents the sequence of the Bovidae, Camelidae, Suidae and Equidae families, while the second represented the Hominidae, Cercopithecidae and Muridae families (Sohrabi et al., 2014).

Although the sequence relationships revealed that the lactoferrin proteins belonged to a highly conserved family (Sohrabi et al., 2014), lactoferrin must have undergone divergent evolution. Genetic distances between groups were

0.242 to 0.601, while the smallest span between taxa was 0.016 (human to gorilla) and the highest was 0.612 (chicken to goat). The polymorphic residue spanned from the amino terminus to the carboxy terminus.

Moreover, lactoferrin is a highly glycosylated protein and the glycosylation pattern seems to vary between species. The lactoferrin of each species exhibits a unique glycosylation pattern that may be responsible for the heterogeneity of the biological properties (Karav et al., 2017). The N-link glycosylation of the "NXS" and "NXT" motives between species also diverge in our dataset.

The genetic distance, phylogeny, polymorphic amino acids, and glycosylation variation should be taken into account in the application of lactoferrin as a feed additive. Feeding with xeno-lactoferrin, i.e. feeding one species with lactoferrin from a different species, especially following long-term and repeated administration, might trigger an immune response that lessens or even obliterates its potency. Data on the immune response to lactoferrin following supplementation is not available yet. The immune response to xeno-lactoferrin might have caused the failure to demonstrate the positive effects of lactoferrin in some studies (Connelly and Erickson, 2016; Cowles et al., 2006; English et al., 2007; Geier et al., 2011; Henry and Alexis, 2009; Shea et al., 2009). Therefore, the best choice should be species specific. A group specific as described in this manuscript is more acceptable than from different group. In other word, chicken lactoferrin or ovotransferrin seems to fit best to poultry, while bovine lactoferrin might work best in pig and goat, beside cattle.

Considering the results of this research, well-designed lactoferrin supplementation should be beneficial to animal production. Lactoferrin feed addition should improve animal production performance by improving lactoferrin intake because its concentration is very low in the milk of various animals (Masson and Heremans, 1971). A reasonable method of production is using DNA-recombinant technology. Lactoferrin has been successfully expressed in various systems, such as bacteria, yeast, fungi, insects, cell lines, mammals, and plants (Garcia-Montoya et al., 2012). Recombinant lactoferrin produced in bacteria or yeast needs to be explored for mass-production which is economically feasible. The effect as feed additive needs to be tested in large population.

5 Conclusion

The lengths of lactoferrin, lactotransferrin or ovotransferrin are 703–711 residues. The phylogeny shows that the lactoferrins of buffalo, cow, goat, pig, camel, and horse form one group; monkey, human, and gorilla form a second group; and dog and cat form third group. Chicken ovotransferrin forms an outgroup. The overall genetic distance was 0.279. Genetic distances between groups were 0.242 to 0.601, while the smallest span between taxa was 0.016 (human to gorilla) and the highest was 0.612 (chicken to goat). The polymorphic and conserved residues span from the amino terminus to the carboxy terminus with 27 conserved cysteine residues. The genetic distance, phylogeny, polymorphic amino acids, and glycosylation variation should be taken into account in the application of lactoferrin as a feed additive. A well-designed lactoferrin supplementation pattern should be beneficial to animal production.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors declare no conflict of interest.

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