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Article

Characterization and antimicrobial efficacy of bovine dermcidin, a novel antimicrobial peptide gene

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Abstract: Description of a novel bovine antimicrobial peptide and its antimicrobial spectrum. RNA isolation and RT-PCR were done from various tissues. DCD peptide was synthesized, and antimicrobial activity was analyzed. Bovine dermcidin gene contains five exons intermittent by 4 introns. Bovine *DCD*-mRNA was 398 bp with ORF 336 bp. Bovine *DCD* was expressed in skin and blood. Analysis of the amino acid compositions revealed that cysteine was repeated 6 times indicating the presence of 3 disulfide bonds that play role in the peptide stability. *Staphylococcus epidermidis*, *Streptococcus bovis*, and *Enterococcus faecalis* were affected by Bovine DCD peptide. Highest antimicrobial effect was at 50 and 100 µg/ml. The effect on *Escherichia coli* and *Candida albicans* was slightly low. In all bacteria, Bovine DCD peptide activity did not affect by varying pH values, but in *Staphylococcus aureus,* the activity was affected greatly at pH 4.5 and 5.5. The optimum salt concentrations were 100 and 50 mM NaCl with all bacterial strains and *E. coli*, respectively. In case of *C. albicans*, the antimicrobial activity of Bovine DCD peptide decreased with increasing the pH value regardless the NaCl concentration. The pH 6.5 of the sweat buffer was the optimum for the Bovine DCD peptide activity.

Keywords: *Bos taurus;* skin; bovine dermcidin; RT-PCR; antimicrobial activity

1. Introduction

The antimicrobial peptides (host-defense peptides**)** are important molecules of innate immune defense with diverse species protecting epithelial barriers. Many antimicrobial peptides show **a** wide antimicrobial spectrum against many pathogens including viruses, bacteria, and fungi [1]; they also act as anti-cancer and aid in wound healing [2]. The antimicrobial peptides have been found in all organisms from bacteria to humans. These antimicrobial peptides have been conserved through evolution across different species with many characteristics of separate AMPs classes. Because of their small size and their high potency, they have been favored through evolution [3,4]. The induction pathways for these peptides in vertebrates, insects, and plants are highly conserved [5,6]. In animals, AMPs are produced at sites that are in contact with microbes, like skin cells or mucosal epithelial cells (oral, respiratory, gastrointestinal, genitourinary, etc.). AMPs in the animal can be constitutively secreted or in response to infection [7].

Ruminant animals (e.g. cattle, sheep, goats, etc.) have a huge number of AMPs that serve as natural innate barriers limiting microbial infectious diseases and proceed as a vital component in reaction to microbial infectious diseases [8,9]. These peptides fluctuate in mechanisms of activity and

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size. There are two groups of AMPs: 1) anionic AMPs, which are a small group present in ruminants, rich in aspartic and glutamic acids, their general antimicrobial activity Gram-negative, Gram-positive bacteria [10] and 2) larger group of cationic AMPs originate in all domestic animals.

There are over 1500 different antimicrobial peptides have been described [11-14] and are being evaluated as possible alternatives to conventional antibiotics. Also, the amphoteric characters of many AMPs enhance their permeation into the lipid of cell membrane causing its destruction and then cell death [15,16].

Notably and anomalous for AMPs, the antimicrobial spectrum of DCD-1L is preserved over a broad pH range even at high salt concentrations, which similar the conditions of human sweat [17]. This notable activity proposed that the functional mechanism of DCD-1L might be different from most other AMPs. There are studies showed a binding of DCD-1L to the bacterial surface and an interaction with bacterial membrane phospholipids [18,19].

In this study, we will describe a novel bovine antimicrobial peptide secrete by skin cells and the spectrum of antimicrobial activity against Gram-positive and negative bacteria as well as fungi.

2. Materials and Methods

2.1. Collection of samples

Different tissues from *Bos taurus* were collected that included skin, spleen, liver, muscle, intestine, kidney and blood. All tissue samples were collected using Allflex Tissue Sampling Units (TSU). Freeze all samples in liquid nitrogen immediately in the field and transfer to -80°C freezer once back in the lab till used.

2.2. RNA expression analysis of dermcidin

RT-PCR was done from various tissues. Isolation of RNA was done using TriFast™ (Peqlab, Erlangen, Germany) in addition; PCR was performed using cDNA from many bovine tissues. The mixture contained 5 µl of the template, 1X *Taq* buffer, 0.4 mM dNTPs, 0.4 µM of each primer and 0.5 µl of *Taq*-polymerase (Fermentas, Germany). The used primers were 5'-GACACACTAGAGACCAGAATCTCC-3' and 5'-TCAAAACATCTGTCCTCCCAC-3', producing a product with ~400 bp. The PCR conditions were 35 cycles at 95ºC for 1 min, 58ºC for 1 min and 72ºC for 80s. The PCR reaction was repeated triple with two negative and one positive control. A ladder of 100-bp (Fermentas, Germany) was used in agarose gel electrophoresis.

2.3. Antimicrobial assay

Bovine DCD peptide was synthesized by FlexPeptide™ Technology (GenScript, USA). The antimicrobial activity of Bovine DCD was analyzed as described by Valore *et al*. [20] using Gram-positive bacteria included *Enterococcus faecalis* (ATCC29212), *Staphylococcus epidermidis* (ATCC1228), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC43330), *Streptococcus bovis* (ATCC49147); while Gram-negative bacteria included *Escherichia coli* (ATCC25922), and the fungus *Candida albicans* (ATCC1021). *E. coli* was grown in LB medium, *S. epidermidis* in Nutrient medium, *S. bovis* in Trypticase soy medium with defibrinated sheep blood, *E. faecalis* and *S. aureus* in Columbia medium, and *C. albicans* in Casein hydrolysate medium. The bacterial and yeast concentrations were determined photometrically. Bacteria and yeast were diluted to a concentration of $2X10^6$ CFU/ml in phosphate buffer. The cells were incubated at 37ºC for 4 h with different peptide concentrations or sweat fractions in 200 µl of phosphate buffer. The microbial cultures were diluted 1:50-500, and 50 µl and 100 µl of the solutions were plated on agar plates in duplicate. At least five plates from each

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experiment were evaluated and the mean number of colonies determined. The bactericidal activity of the tested reagents represented the percentage of cells that killed and was expressed as:

$$\text{Bactericidal activity} = 1 - \frac{\text{number of cells survival after peptide incubation}}{\text{number of cell survival after control peptide incubation}} X\ 100$$

To examine the activity profile of Bovine DCD and sweat fraction that contained Bovine DCD, the microorganisms were incubated for 4 h with 10 μg/ml of Bovine DCD or sweat fraction under the following conditions: phosphate buffer (10 mM) + either 25, 100 or 150 mM sodium chloride, phosphate buffer (10 mM) at pH 4.5, 5.5, 6.5 or 7.5; sweet buffer (40 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM Na-dihydrogen phosphate) at pH 5.5 or 6.5. The antimicrobial activity was assessed as outlined above.

3. Results

The discovered bovine dermcidin gene was not published before, we detect the locus and the sequence of the gene using *in silico* determination based on conserved synteny between human chromosome 12, bovine chromosome 5, monkey chromosome 11 and chimpanzee chromosome 12 [21-23]. By alignment the human dermcidin gene in the cattle genome, we detect the sequence which was aligned in the cattle genome and by analysis the segment we find the sequence of bovine DCD gene (Table S1 and Figure S1).

3.1. Analysis of bovine dermacidin nucleotid

The gene was designated dermcidin (Bovine *DCD*) that had no homology to any bovine published gene sequence. Sequencing of the products identified a full-length Bovine *DCD*-cDNA of 398 bp with an open reading frame of 336 bp (Figure 1). The full length of dermcidin gene was 2205 bp, containing five exons intermittent by 4 introns varying in size, in which the exon 1 was 59 bp, exon 2 was 23 bp, exon 3 was 112 bp, exon 4 was 90 bp, exon 5 was 52 bp and finally 3 bp stop codon (Figure 1), when compared with the PCR fragment Bovine *DCD*-cDNA sequence. All the introns splice junctions corresponded to the GT–AG rule. The Genebank accession number for bovine *DCD* gene sequence we identified is AB932628.

Figure 1. Structure of the bovine dermcidin gene (bovine *DCD*) secreted by skin of *Bos taurus* and has accession number AB932628.

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3.2. Analysis of bovine dermacidin peptide

To determine the expression profile of Bovine *DCD*, RNA from different bovine tissues were tested using reverse transcriptase-PCR (RT-PCR). Bovine *DCD* was expressed in bovine skin tissue and blood (Figure S2). However, expression was not detected in any other tissues (spleen, liver, muscle, intestine, and kidney). These results indicated that Bovine *DCD* expression was restricted to cells in the bovine skin. By analyzing the amino acids sequence of the Bovine DCD peptide indicated that is composed of 18 different amino acids repeated to produce a peptide with 112 amino acids. The most abundant amino acids were proline and leucine (repeated by 15 times each) and the rarest amino acids were histidine, asparagine, and tryptophan (represented by one time each). Cysteine was repeated 6 times which indicated that there are 3 disulfide bonds that play a role in the stability of the peptide (Table 1). The peptide sequence was applied to SignalP-4.1 online program (http://www.cbs.dtu.dk/services/SignalP/) to determine the signal peptide length that was the first 21 amino acids (Figure 2). Also, the predicted Bovine DCD-peptide sequence was applied to Compute pI/Mw (http://web.expasy.org/cgi-bin/compute_pi/pi_tool) to determine the theoretical isoelectric point and molecular weight of the peptide that was 9.24 for pI and 11.91 kDa for Mw.

Table 1: Amino acids composition of the deduced *Bos tautrus* DCD-AMP

Amino acid	Single letter	Number	Type of amino acid
Methionin	M	3	Non polar (hydrophobic)
Arginine	R	5	+ve
Leucine	L	15	Non polar (hydrophobic)
Threonine	T	4	Polar (hydrophilic)
Alanine	A	12	Non polar (hydrophobic)
Proline	P	15	Non polar (hydrophobic)
Glutamic acid	E	7	-ve
Cysteine	C	6	Polar (hydrophilic)
Glycine	G	10	Non polar (hydrophobic)
Isoleucine	I	2	Non polar (hydrophobic)
Lysine	K	8	+ve
Phenylalanine	F	5	Non polar (hydrophobic)
Serine	S	8	Polar (hydrophilic)
Histidine	H	1	+ve
Glutamine	Q	7	Polar (hydrophilic)
Asparagine	N	1	Polar (hydrophilic)
Tryptophan	W	1	Non polar (hydrophobic)
Valine	V	2	Non polar (hydrophobic)

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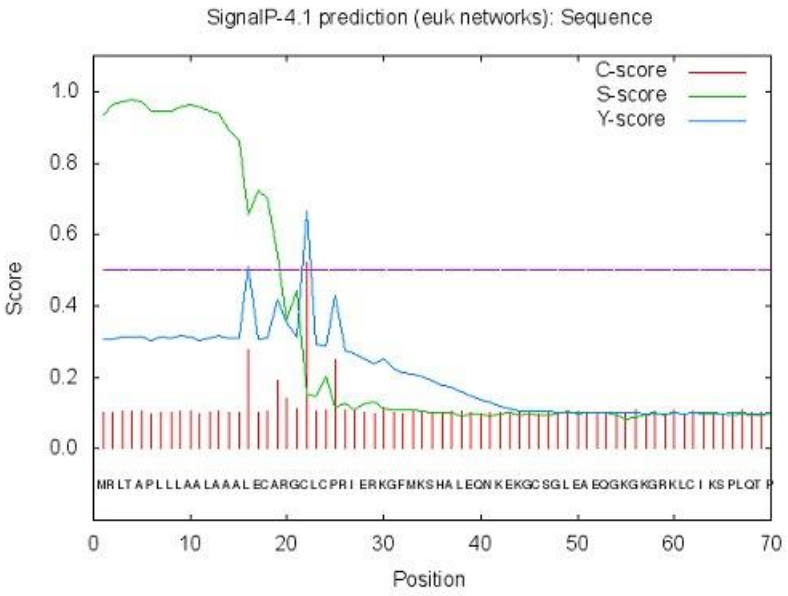

*MRLTAPLLLAALAAALECARG*CLCPRIERKGFMKSHALEQNKEKGCSGLEAEQGKGKGRKLCIKSP

LQTPPSPPWESGACPAPFPQLPSFQLPVLSSQTPLAPFTPVGGQMF

Figure 2. The signal peptide length was determined using online SignalP-4.1 euk predictions (http://www.cbs.dtu.dk/services/SignalP/).

3.3. Antimicrobial activity of bovine dermacidin peptide

The synthesized Bovine DCD peptide had a broad antimicrobial activity against gram +ve, gram –ve bacteria and yeast fungus. For G +ve bacteria, the most affected species were *S. epidermidis*, *S. bovis* and *E. faecalis*, respectively, which the antimicrobial effect was increased with peptide concentration increased. The highest antimicrobial effect was achieved at 50 and 100 µg/ml, where no significant difference between the two concentrations in the effect. The methicillin-resistant *S. aureus* (MRSA) behaved the same behavior but the maximum antimicrobial effect was achieved at 50 µg/ml, and then decreased at 100 µg/ml. For G –ve bacterium (*E. coli*) the antimicrobial effect was slightly less than G +ve bacteria. In case of *C. albicans*, it affected with the antimicrobial peptide, where no effect at 1 µg/ml and the inhibitory effects starts at 5 µg/ml increase reaching the maximum at 100 µg/ml (Figure 3).

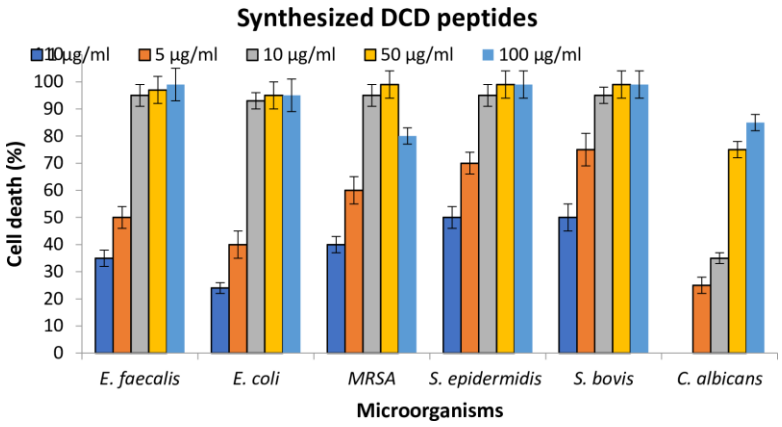

Figure 3. Antimicrobial efficacy (cell death) of various concentrations of synthesized Bovine DCD peptides against selected microorganisms.

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To study the antimicrobial efficacy of the Bovine DCD peptide, we study the antimicrobial activity using different pH values with or without NaCl concentrations. In all bacterial strains used the varying of pH value did not affect in the activity of Bovine DCD peptide, except in *S. aureus* the antibacterial activity affected greatly at pH 4.5 and 5.5, where the cell death percent is less than 30% (Figure 4). In presence of NaCl concentrations, the antimicrobial activity of Bovine DCD peptide did not affect by varying the salt concentrations, where 100 mM NaCl was the optimum concentration which gave the highest antibacterial activity in all bacterial strains except *E. coli* in which the antibacterial activity was affected greatly after 50 mM NaCl concentration, where the optimum NaCl concentration was 50 mM NaCl. The behavior of *C. albicans* was on contrary to the bacterial behavior, where the antimicrobial activity of Bovine DCD peptide decreased with increasing the pH in absence and presence of NaCl concentrations. In general pH 6.5 of the sweat buffer was the most suitable for increasing the activity of the Bovine DCD peptide in all bacterial strains (Figure 4).

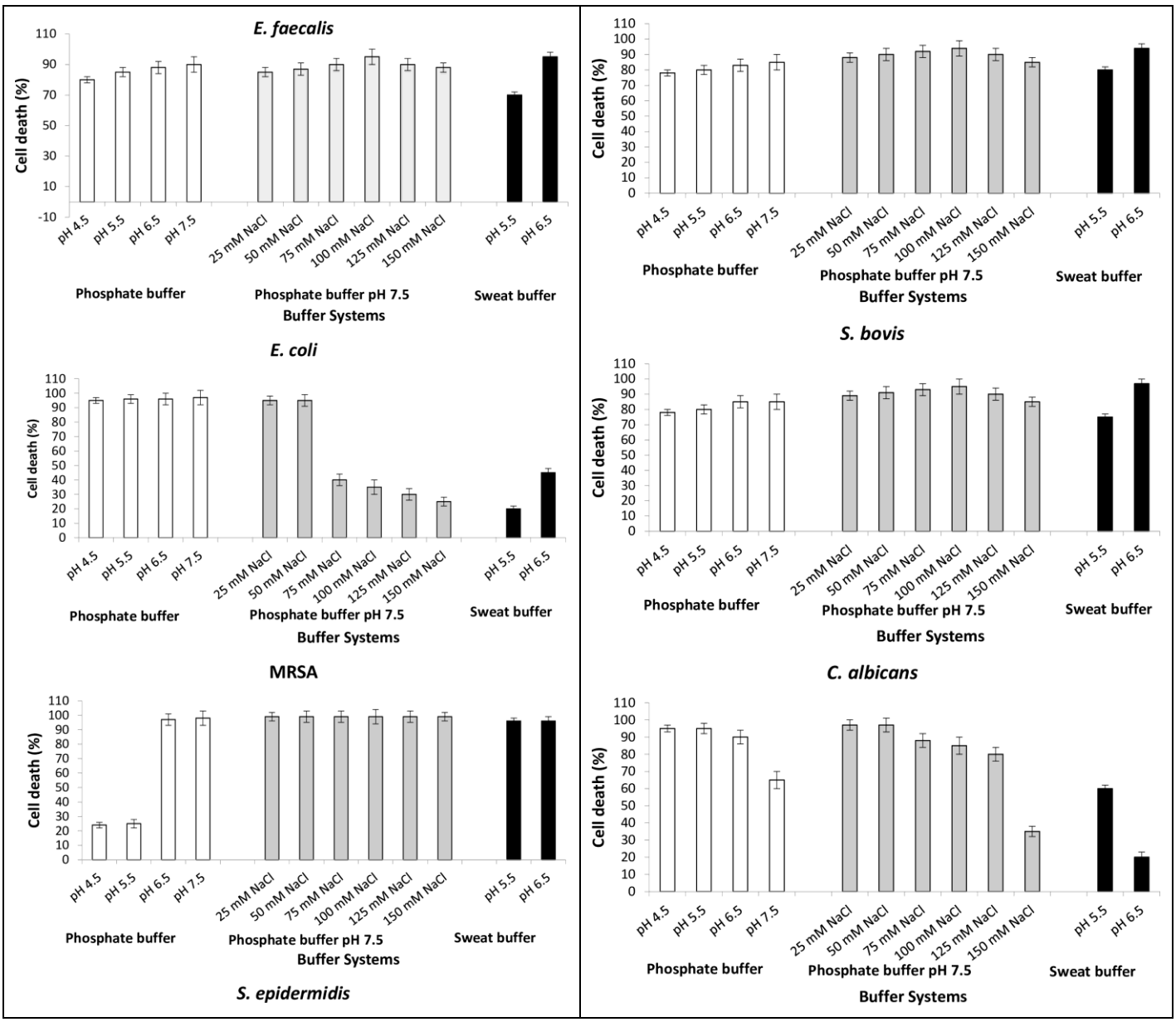

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Figure 4. Antimicrobial efficacy of the bovine DCD secreted from skin cells of *Bos taurus* on selected microorganisms, using different pH values of phosphate buffer, pH 7 of phosphate buffer amended with different concentrations of salt, and different pH values of sweat buffer.

4. Discussion

The discovery of antimicrobial peptides in animals is expanding and advances in this field are expected. It will be likely to find the new genes encoding antimicrobial peptides in sequenced animal genomes [24].

The full length of dermcidin gene was 2205 bp, containing five exons intermittent by 4 introns varying in size, in which the exon 1 was 59 bp, exon 2 was 23 bp, exon 3 was 112 bp, exon 4 was 90 bp, exon 5 was 52 bp and finally 3 bp stop codon, when compared with the PCR fragment Bovine *DCD*-cDNA sequence. Sequencing of the products identified a full-length Bovine *DCD*-cDNA of 398 bp with an open reading frame of 336 bp

These findings agreed with Schittek *et al*. [17] who determined that the human *DCD* gene contains 5 exons and is expressed as a single transcript. Dermcidin was identified for the first time in human as a gene specifically expressed in sweat glands, coding for a 110-amino acid protein (Bovine DCD).

Bovine *DCD* was expressed in bovine skin tissue and blood only. However, expression was not detected in any other tissues (spleen, liver, muscle, intestine, and kidney). These results indicated that Bovine *DCD* expression was restricted to cells in the bovine skin. Bovine DCD peptide composed of 18 different amino acids repeated to produce a peptide with 112 amino acids. The most abundant amino acids were proline and leucine (repeated by 15 times each) and the rarest amino acids were histidine, asparagine, and tryptophan (represented by one time each). Cysteine was repeated 6 times which indicated that there are 3 disulfide bonds that play a role in the stability of the peptide. The signal peptide length was the first 21 amino acids. Also, the theoretical isoelectric point and molecular weight of the peptide that was 9.24 for pI and 11.91 kDa for Mw.

The product of full-length of the human dermcidin gene is 110 amino acids, with 19 amino acids as N-terminal signal peptide [25]. The 110 amino acids precursor is processed proteolytically in sweat, giving rise to many truncated DCD peptides differing in length and charge [26-28].

Eccrine are the most developed and abundant glands in humans. They are distributed in all over the body surface. On the other hand, in cow, donkey, horse, camel and canid species, apocrine glands are distributed on all over the surface of the animal body and have evaporative cooling functions. Eccrine glands secret sweat with odorless, consisting of mainly from water, with amino acids, proteins, ammonia, urea, lactic acid and small traces of salts. Apocrine glands secrete sweat that consists of proteins, lipids, and steroids [29]. The expression of human dermcidin (*DCD*) is localized in skin cells, where is expressed in eccrine sweat glands constitutively, secreted into the sweat and transported to the skin surface [17].

The synthesized Bovine DCD peptide had a broad antimicrobial activity against All microorganisms tested in this study. The most affected species were *S. epidermidis*, *S. bovis* and *E. faecalis*, respectively, which the antimicrobial effect was increased with peptide concentration increased. The highest antimicrobial effect was achieved at 50 and 100 µg/ml, where no significant difference between the two concentrations in the effect. *S. aureus* (MRSA) behaved the same behavior but the maximum antimicrobial effect was achieved at 50 µg/ml, and then decreased at 100 µg/ml.

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For *E. coli* the antimicrobial effect was slightly low. In case of *C. albicans*, it affected with the antimicrobial peptide, where no effect at 1 μg/ml and the inhibitory effects starts at 5 μg/ml increase reaching the maximum at 100 μg/ml.

Anionic antimicrobial peptides which are a small group present in ruminants, rich in aspartic and glutamic acids, their general antimicrobial activity Gram-negative and Gram-positive bacteria [10,30]. The fraction of DCD peptide in sweat has antimicrobial spectrum against a wide range of pathogenic microorganisms. Cattle have apocrine sweat glands in which each hair fiber associated with one gland [31,32].

The major dermcidin peptide fragment in sweats is the DCD-1L with 48-mer and has net charge -2 that is anionic and capable of to kill/inhibit microbes like *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, rifampin- and isoniazid-resistant *Mycobacterium tuberculosis*, methicillin-resistant *S. aureus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Salmonella thyphimurium*, *Pseudomonas putida* and *Candida albicans* [17,25,33,34].

The antimicrobial activity using different pH values with or without NaCl concentrations were studied. In all bacterial strains used the varying of pH value did not affect in the activity of Bovine DCD peptide, except in *S. aureus* the antibacterial activity affected greatly at pH 4.5 and 5.5, where the cell death percent is less than 30%. The NaCl concentrations 100 mM was the optimum which gave the highest antibacterial activity in all bacterial strains except *E. coli* in which the antibacterial activity was affected greatly after 50 mM NaCl concentration, where the optimum NaCl concentration was 50 mM NaCl. The behavior of *C. albicans* was on contrary to the bacterial behavior, where the antimicrobial activity of Bovine DCD peptide decreased with increasing the pH in absence and presence of NaCl concentrations. In general pH 6.5 of the sweat buffer was the most suitable for increasing the activity of the Bovine DCD peptide in all bacterial strains.

Cattle sweat has high contents of total protein nitrogen and the inorganic salt and relatively high urea content [35]. The human DCD-1L has a broad spectrum of antimicrobial activity over a wide pH range and in high salt concentrations [36]. The antimicrobial activity of DCD-1L is maintained over a broad pH range and at high salt concentrations that resemble the conditions in human sweat [17].

5. Conclusions

The bovine DCD gene expressed in skin cells and the bovine DCD peptide secreted into the sweat which had high antimicrobial activities against Gram-positive and negative bacteria as well as yeast-like fungus. The results revealed that the bovine DCD peptide activity did not affect by different salt concentrations, this means that the bovine DCD peptide was adapted to work in all salt concentrations that secreted in the sweat.

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Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1,

Figure S1. Phylogenetic tree showed the relation between the discovered bovine dermcidin mRNA (this study) and the other dermcidin genes in different organisms.

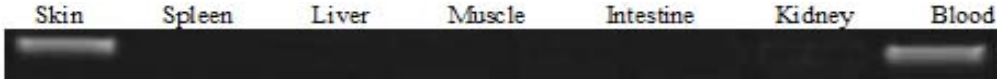

Figure S2. RT-PCR analysis of various tissues. As shown bovine DCD expression was restricted to cells in the skin.

Table S1. Comparative genome map of Chromosome 12 (*Homo sapiens*), Chromosome 12 (*Pan troglodytes*), Chromosome 11 (*Macaca mulatta*) and Chromosome 5 (*Bos taurus*), the compared segments was mentioned between brackets.

Organism	Gene		Locus
	Name	Abbreviation	
Homo sapiens (Human)	Neuronal differentiation 4	NEUROD4	Chromosome 12, NC_000012.12 (55019945..55030017)
Pan troglodytes (Chimpanzee)	Neuronal differentiation 4	NEUROD4	Chromosome 12, NC_036891.1 (34055442..34065514, complement)
Macaca mulatta (Monkey)	Neuronal differentiation 4	NEUROD4	Chromosome 11, NC_027903.1 (53721646..53731727)
Bos taurus (Cattle)	Neuronal differentiation 4	NEUROD4	Chromosome 5, NC_037332.1 (59882602..59920743, complement)
Homo sapiens (Human)	Thymocyte expressed, positive selection associated 1	TESPA1	Chromosome 12, NC_000012.12 (54948019..54984746, complement)
Pan troglodytes (Chimpanzee)	Thymocyte expressed, positive selection associated 1	TESPA1	Chromosome 12, NC_036891.1 (34100708..34136106)
Macaca mulatta (Monkey)	Thymocyte expressed, positive selection associated 1	TESPA1	Chromosome 11, NC_027903.1 (53651281..53686091, complement)
Bos taurus (Cattle)	Thymocyte expressed, positive selection associated 1	TESPA1	Chromosome 5, NC_037332.1 (59925012..59960265)
Homo sapiens (Human)	Mucin like 1	MUCL1	Chromosome 12, NC_000012.12 (54854515..54858393)
Pan troglodytes (Chimpanzee)	Mucin like 1	MUCL1	Chromosome 12, NC_036891.1 (34226741..34230956, complement)
Macaca mulatta (Monkey)	Mucin like 1	MUCL1	Chromosome 11, NC_027903.1 (53526781..53530653)
Bos taurus (Cattle)	Mucin-like 1	MUCL1	Chromosome 5, NC_037332.1 (25215043..25220249, complement)

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Homo sapiens (Human)	Dermcidin	DCD	Chromosome 12, NC_000012.12 (54644591..54648493, complement)
Pan troglodytes (Chimpanzee)	Dermcidin	DCD	Chromosome 12, NC_036891.1 (34435763..34439668)
Macaca mulatta (Monkey)	Dermcidin	DCD	Chromosome 11, NC_027903.1 (53338985..53343039, complement)
Bos taurus (Cattle)	Dermcidin	DCD	chromosome 5, NC_037332.1 (25435277.. 25437603)
Homo sapiens (Human)	Lacritin	LACRT	Chromosome 12, NC_000012.12 (54630839..54634879, complement)
Pan troglodytes (Chimpanzee)	Lacritin	LACRT	Chromosome 12, NC_036891.1 (34449360..34453441)
Macaca mulatta (Monkey)	Lacritin	LACRT	Chromosome 11, NC_027903.1 (53324690..53328815, complement)
Bos taurus (Cattle)	Lacritin	LACRT	chromosome 5, NC_037332.1 (25446731..
Homo sapiens (Human)	glycosylation dependent cell adhesion molecule 1	GLYCAM1	Chromosome 12, NC_000012.12 (54608187..54610462, complement)
Pan troglodytes (Chimpanzee)	-	-	-
Macaca mulatta (Monkey)	-	-	-
Bos taurus (Cattle)	glycosylation-dependent cell adhesion molecule 1	GLYCAM1	Chromosome 5, NC_037332.1 (25478966..25481870)
Homo sapiens (Human)	protein phosphatase 1 regulatory inhibitor subunit 1A	PPP1R1A	Chromosome 12, NC_000012.12 (54579240..54588659, complement)
Pan troglodytes (Chimpanzee)	protein phosphatase 1 regulatory inhibitor subunit 1A	PPP1R1A	Chromosome 12, NC_036891.1 (34495553..34503977)
Macaca mulatta (Monkey)	protein phosphatase 1 regulatory inhibitor subunit 1A	PPP1R1A	Chromosome 11, NC_027903.1 (53271995..53281127, complement)
Bos taurus (Cattle)	protein phosphatase 1 regulatory inhibitor subunit	PPP1R1A	Chromosome 5, NC_037332.1 (25506930..25514982)
Homo sapiens (Human)	phosphodiesterase 1B	PDE1B	Chromosome 12, NC_000012.12 (54549393..54579239)
Pan troglodytes (Chimpanzee)	phosphodiesterase 1B	PDE1B	Chromosome 12, NC_036891.1 (34504986..34535264, complement)
Macaca mulatta (Monkey)	phosphodiesterase 1B	PDE1B	Chromosome 11, NC_027903.1 (53241661..53271924)
Bos taurus (Cattle)	phosphodiesterase 1B	PDE1B	Chromosome 5, NC_037332.1 (25515721..25542387, complement)

Author Contributions: Conceptualization, N.S. and T.M.; methodology, N.S. and T.M.; formal analysis, N.S. and T.M.; investigation, N.S. and T.M.; resources, N.S. and T.M.; data curation, N.S. and T.M.; writing—original draft preparation, N.S.; writing—review and editing, T.M.

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