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Research article

Semi-quantitative comparison of lactophorin in bovine and caprine milks using liquid chromatography/tandem mass spectrometry

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Abstract: Lactophorin, also known as glycosylation-dependent cell adhesion molecule 1, is a phosphorylated glycoprotein found in bovine and caprine milks. This protein is located on the milk fat globule membrane (MFGM), with only a weak association to it. Bovine lactophorin exhibits anti-rotavirus and antibacterial activities and provides stable emulsifying properties during milk processing. In this study, a semi-quantitative method for comparing lactophorin levels in bovine and caprine dairy products was developed using liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis of LPLSILK, a shared common proteotypic peptide between the two species. A single prominent LPLSILK peak was clearly detected on multiple reaction monitoring (MRM) chromatograms of tryptic digests from bovine and caprine milk protein extracts, enabling straightforward cross-species comparison based on normalized peak areas. Lactophorin levels were evaluated as normalized LPLSILK peak areas per unit volume (milk) or per unit weight (milk powder), without the use of a stable isotope-labeled internal standard or an external calibration curve. Using this semi-quantitative framework, no statistically significant difference was observed between bovine and caprine milks ($P = 0.39$). The method was also applicable to milk powders. Future in vitro and in vivo studies are warranted to further examine the biological activities of caprine lactophorin, particularly its potential antimicrobial and antiviral properties.

Keywords: lactophorin; milk; cow; goat; targeted proteomics

1. Introduction

Rotavirus is the leading cause of acute gastroenteritis in children [1] and contributes to high infant mortality rates in developing countries [2]. Globally, the virus accounts for 130 million childhood infections and over 200,000 deaths each year [3]. To prevent rotavirus disease, vaccines have been introduced in more than 95 countries and have considerably reduced diarrheal mortality in young children [4].

Food-derived substances have been reported to exert antiviral effects against rotaviruses [3]. Resveratrol, a polyphenolic compound present in berries and nuts, exhibits anti-rotavirus activity in cellular and animal experiments. Thirty-four flavonoids likewise demonstrate anti-rotavirus activity. In addition to polyphenols, vitamin D suppresses rotavirus infection. Among the proteins present in food, lactophorin—also known as glycosylation-dependent cell adhesion molecule 1—from bovine milk has been reported to inhibit rotavirus [5].

Lactophorin is present on the milk fat globule membrane (MFGM) and is weakly associated with it. Because this protein inhibits lipase activity in milk, it has been suggested to play a role in limiting the access of lipases to the milk fat globule lipid core and interfering with spontaneous lipolysis in milk [6]. In addition to its inhibitory effect on rotavirus, lactophorin has a pore-forming ability at its C-terminal site; therefore, it is thought that this site interacts with lipid bilayers, such as bacterial membranes. The corresponding synthetic peptide suppresses the growth of gram-negative and gram-positive bacteria [7]. Lactophorin has stable emulsifying properties during milk processing [8].

In addition to bovine milk, lactophorins are also present in the milk of goat, sheep, camel, and llama [9]. The concentration of lactophorin in bovine milk is reported to be 0.3–0.37 g/L [6,10]. The concentration in camel milk varies from 2.7 to 6.8 g/L during the first eight days of lactation [8]. Sørensen et al. [11] reported the western blot analysis of bovine and caprine milk samples using antibodies against bovine lactophorin. In their study, the staining intensities in the lanes containing these milk samples were similar, but quantitative data was not provided. Zhang et al. [12] investigated the changes in bovine, caprine, and camel milk proteins after freezing, pasteurization, and spray drying using a dimethyl-labeling proteomic technique followed by liquid chromatography/tandem mass spectrometry (LC/MS/MS). In their study, the concentration of lactophorin in unheated milk serum differed among cow, goat, and camel. However, they did not present comparative data on the concentrations in whole milk samples.

Targeted proteomics using LC/MS/MS provides high accuracy and reproducibility in protein quantification. Moreover, this technique does not require the laborious construction of antibodies for detecting the target protein [13]. In targeted proteomics, fragment peptides are generated from the target protein using a protease (e.g., trypsin). After protein digestion, proteotypic peptides with sequences unique to the target protein are analyzed using LC/MS/MS. Therefore, the molar amount of proteotypic peptides after complete digestion represents the molar amount of the protein in the sample [14]. Because the signal intensity of a proteotypic peptide reflects the abundance of the parent protein, peptide peak areas can be used for comparative evaluation of protein levels across samples. To date, no quantitative method has been established that enables direct cross-species comparison of lactophorin abundance between cow and goat using a shared proteotypic peptide. Therefore, the development of a cross-species-comparable LC/MS/MS approach is essential for evaluating similarities and differences in lactophorin across dairy products. The present study aimed to establish a semi-quantitative LC/MS/MS approach using a shared proteotypic peptide, LPLSILK, to enable direct comparative evaluation of lactophorin abundance in bovine and caprine milks and milk powders.

2. Materials and methods

2.1. Materials

All reagents used for protein extraction, enzymatic digestion, and LC/MS/MS analysis were of analytical or LC/MS grade. Ammonium bicarbonate, dithiothreitol, and trichloroacetic acid were sourced from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Guanidinium chloride and β-mercaptoethanol were supplied by Sigma-Aldrich (St. Louis, USA). Iodoacetamide was obtained from FUJIFILM Wako Pure Chemical Corporation and Sigma-Aldrich. MS-grade trypsin was supplied by Thermo Fisher Scientific (Waltham, USA). Ultrapure water was prepared using a Milli-Q purification system (Merck, Darmstadt, Germany).

Commercial samples consisted of bovine liquid milk from three different brands (Cows A, B, and C), caprine liquid milk from three brands (Goats D, E, and F), whole milk powders derived from bovine (Cows G and H) and caprine (Goats J and K) sources, and skimmed milk powders from bovine (Cow I) and caprine (Goat L) origins. All products were obtained through local retail and online sources. Based on the label information, Cows A and B were produced from 100% raw milk of Japanese origin, sterilized for 2 s at 130 °C, and contained at least 8.3% and at least 8.4% solid-not-fat and at least 3.5% and at least 3.7% milk fat, respectively; Cow C was produced from 100% raw milk of Japanese origin, sterilized for 30 min at 63 °C, and contained at least 8.3% solid-not-fat and at least 3.5% milk fat; Goats D and F were produced from 100% raw milk of Japanese origin, sterilized for 30 min at 65 °C, and contained 8.2% and at least 7.5% solid-not-fat and 3.1% and at least 2.5% milk fat, respectively; Goat E was produced from 100% raw milk of Japanese origin and contained at least 8.0% solid-not-fat and at least 3.0% milk fat; Cows G, H, and I were produced from 100% raw milk of Japanese origin; and Goats J, K, and L originated from the Netherlands.

The primary amino acid sequences of bovine lactophorin (UniProt accession number: P80195) and caprine lactophorin (UniProt accession number: P81447) were obtained from the UniProt database (https://www.uniprot.org/), and subjected to in silico tryptic digestion (PeptideCutter, https://web.expasy.org/peptide_cutter/). Candidate peptide was screened based on length, absence of labile residues, and suitability for multiple reaction monitoring (MRM) analysis. A single tryptic peptide, LPLSILK, which is conserved between bovine and caprine lactophorins, was selected as a common analytical target and synthesized (GenScript Biotech Corporation, Nanjing, China, a purity of 95%) for method development.

2.2. Sample preparation

Milk proteins were isolated using a precipitation-based workflow designed to remove lipids and low-molecular-weight interfering substances prior to LC/MS/MS analysis [15]. Liquid milk (0.1 mL) and milk powder (20 mg) samples were precipitated by adding 1 mL of 10% (w/v) trichloroacetic acid in acetone mixed with 2% (v/v) β-mercaptoethanol, followed by overnight incubation at -20 °C. After incubation, the mixtures were centrifuged at 14,000 $\times g$ for 5 min at 4 °C, and the resulting supernatants were removed. The pellets were subsequently washed by adding 1 mL of pre-chilled acetone (-20 °C) and centrifuged again under the same conditions, after which the supernatants were discarded. The samples were subjected to two additional washes with cold acetone and then dried.

The precipitated proteins were re-suspended in 100 µL of a chaotropic solution composed of 50 mM ammonium bicarbonate and 6 M guanidinium chloride [15]. Reduction was carried out by adding 5 µL of 200 mM dithiothreitol in 50 mM ammonium bicarbonate, followed by boiling for 10 min.

Alkylation was then performed by adding 4 μL of 1 M iodoacetamide in 50 mM ammonium bicarbonate and incubating the samples for 1 h at room temperature in the dark. To quench excess iodoacetamide, 40 μL of 200 mM dithiothreitol in 50 mM ammonium bicarbonate was added, and the mixtures were incubated for an additional 1 h at room temperature. The samples were subsequently diluted with 50 mM ammonium bicarbonate (851 μL). Aliquots (50 μL) of these solutions were further diluted with 450 μL of the same buffer. Proteolytic digestion was initiated by mixing 50 μL of the diluted samples with 50 μL of 5 μg/mL trypsin in 50 mM ammonium bicarbonate and incubating for 24 h at 37 °C. Enzymatic digestion was terminated by addition of 0.5 μL of formic acid, and the digests were stored at -20 °C until LC/MS/MS analysis.

2.3. LC/MS/MS analysis

Targeted LC/MS/MS analysis was performed using an ultra-performance liquid chromatography system (ACQUITY UPLC, Waters, Milford, USA) coupled to a triple quadrupole mass spectrometer (XEVO TQD, Waters). The mass spectrometer was operated under the following conditions: A capillary voltage of 3 kV, cone gas flow of 50 L/h, desolvation gas flow of 800 L/h, desolvation temperature of 300 °C, and source temperature of 150 °C. To optimize signal intensity, the cone voltage and collision energy for LPLSILK were optimized by direct infusion of the synthesized peptide, and its product-ion spectrum was acquired. The MRM transitions calculated using Skyline software (https://skyline.ms/project/home/begin.view), cone voltages, and collision energies are listed in Table 1. The dwell time was set to 400 ms for quantification transition (392.3 to 335.7), whereas the dwell times of 5 ms were applied for identification (392.3 to 573.4 and 392.3 to 670.4).

Table 1. MRM transition, cone voltage, and collision energy for LPLSILK analysis.

m/z Precursor ion (z = +2)	Product ion	Cone voltage (V)	Collision energy (V)
392.3	***335.7*** (z = +2, y_6)	30	14
392.3	573.4 (z = +1, y_5)	30	14
392.3	670.4 (z = +1, y_6)	30	14

*Note: The *m/z* values of the precursor and product ions were calculated using the Skyline software. Italicized transitions in bold were used to quantify LPLSILK. Other transitions were used for identification.

Aliquots (2 μL) of trypsin-digested samples were injected onto a reversed-phase C18 column (ACQUITY UPLC Peptide CSH C18 Column, 130Å, 1.7 μm, 1 mm × 150 mm, Waters) maintained at 40 °C. Chromatographic separation was carried out at a flow rate of 70 μL/min using a binary gradient composed of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) as follows: 3% B at 0 and 0.5 min, 55% B at 2 min, 70% B at 9 min, 98% B at 9.2 and 9.5 min, and 3% B at 10 min. Data acquisition and peak integration were conducted using MassLynx software (Waters) and Skyline software, respectively. Each milk and milk powder brand was analyzed in triplicate. Data were evaluated as semi-quantitative (relative) abundance based on normalized LPLSILK peak areas per unit volume (milliliters of milk) or per unit weight (grams of milk powder). No stable isotope-labeled internal standard and no external calibration curve were used; therefore, the results are reported as relative, peak-area–based comparisons rather than absolute concentrations. Statistical comparisons between bovine and caprine samples were performed using an unpaired *t*-test (Excel, Microsoft,

Redmond, USA).

3. Results and discussion

3.1. Semi-quantitative comparative analysis

Based on the in silico selection criteria for proteotypic peptides, such as a peptide length between 6 and 16 amino acids, no M or C residues, and no posttranslational modifications [16,17], LPLSILK was selected from all tryptic peptide fragments to compare lactophorin levels in bovine and caprine dairy products. Because LPLSILK is a common tryptic peptide generated from both bovine and caprine lactophorins, whose sequences are located at amino acids 83–89 and 84–90, respectively (Figure 1), this peptide can be used for the semi-quantitative comparison of lactophorins between cow and goat. Bär et al. [10] reported an LC/MS/MS method that enables the simultaneous quantification of 20 bovine milk proteins. In their study, they quantified bovine lactophorin using LPLSILK as a proteotypic peptide. Considering the above-mentioned points, LPLSILK was judged to be appropriate for the semi-quantitative comparison of bovine and caprine lactophorins.

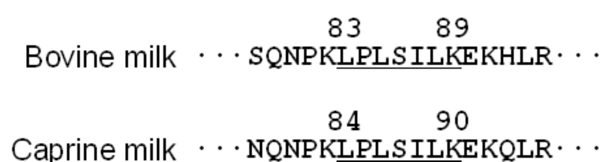

Figure 1. Amino acid sequences of lactophorin derived from bovine and caprine species. The proteotypic peptide selected for semi-quantitative comparison is highlighted by underlining.

After optimizing the MS/MS parameters using the synthesized LPLSILK, the product-ion spectrum originating from the doubly protonated precursor ion of LPLSILK was measured (Figure 2). Because the doubly charged y_6 ion was observed with the stronger intensity than single-charged y_5 and y_6 ions, the MRM transitions were determined as follows: the transition (*m/z* 392.3 to 335.7) and other ion transitions were chosen for the quantitative measurement of LPLSILK and its identification, respectively (Table 1).

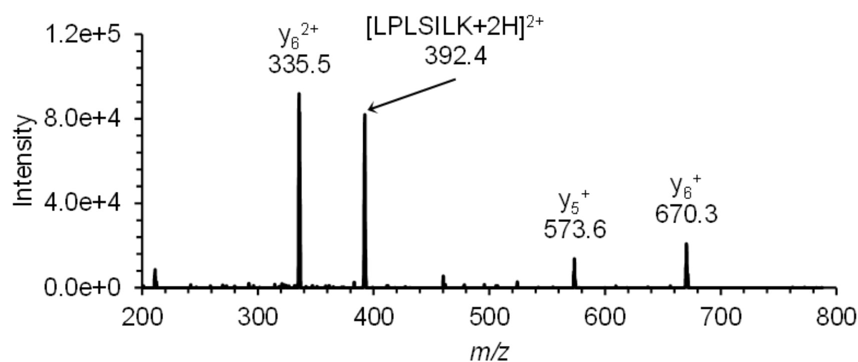

Figure 2. Product-ion spectrum originating from doubly charged LPLSILK precursor ion.

Figure 3 depicts typical chromatograms detected via the transition (*m/z* 392.3 to 335.7) after injecting trypsin digest from bovine (Figure 3(A)) and caprine (Figure 3(B)) milk protein extracts. A single prominent peak of LPLSILK with retention time of 7.1–7.2 min was clearly observed on both chromatograms, which easily enabled semi-quantitative comparison of bovine and caprine lactophorins by comparing the peak area values of LPLSILK derived from their lactophorins.

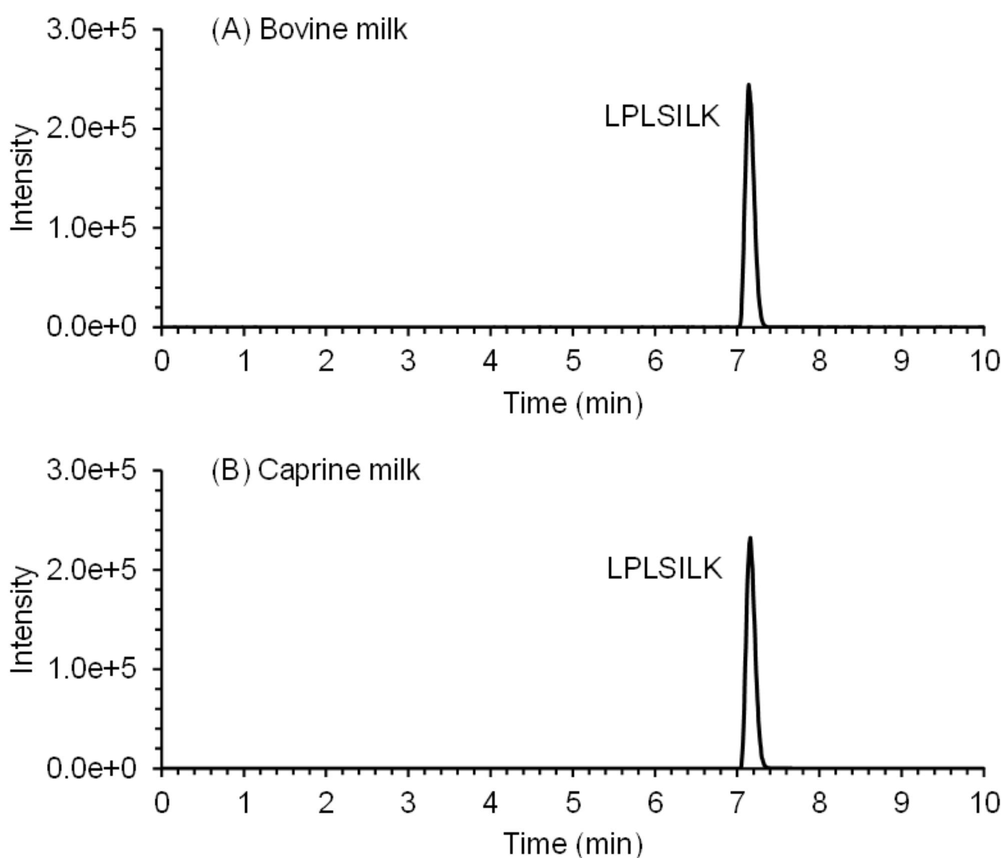

Figure 3. MRM chromatograms obtained from tryptic digests of bovine (A) and caprine (B) milk samples. The traces represent signals acquired using the MRM transition (*m/z* 392.3 to 335.7, Table 1) for LPLSILK analysis.

3.2. Comparison of lactophorin levels

Figure 4 shows normalized LPLSILK peak areas per unit volume (milliliters of milk) of six brands of commercial bovine and caprine milk. Under the present semi-quantitative framework, normalized peak areas were comparable among brands within each species, and no statistically significant difference was observed between bovine and caprine milks (P = 0.39).

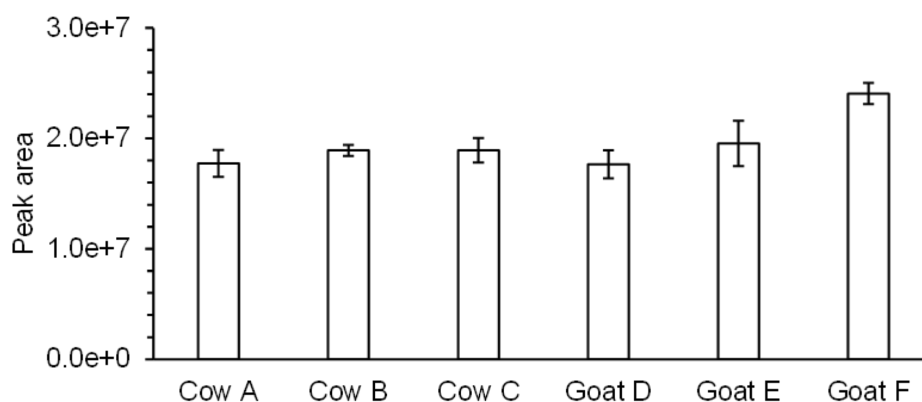

Figure 4. Semi-quantitative comparison of lactophorin levels in commercial bovine and caprine milks. Values are expressed as normalized LPLSILK peak areas (arbitrary units) per unit volume (milliliters of milk) and shown as mean ± standard deviation, n = 3.

In western blot analysis of bovine and caprine milk samples with antibodies against bovine lactophorin, the staining intensities of these samples were similar [11]. The result of the experiment (Figure 4) using targeted proteomic technology matched that of the western blot analysis. This suggests that caprine lactophorin abundance is comparable to that of bovine lactophorin, which has been reported to be 0.3–0.37 g/L [6,10]. However, this result does not agree with the results of Zhang et al. [12], who found that the concentration of lactophorin in unheated milk serum differed among cow, goat, and camel. One possible reason for this discrepancy is the difference in the measured samples (whole milk and unheated milk serum).

Subsequently, lactophorin levels were examined in six commercially available bovine and caprine milk powder products. The normalized LPLSILK peak area per unit weight (grams of milk powder) are presented in Figure 5. Skimmed milk powders showed higher normalized LPLSILK peak areas than whole milk powders, which may reflect enrichment of lactophorin in the skim fraction during the defatting process.

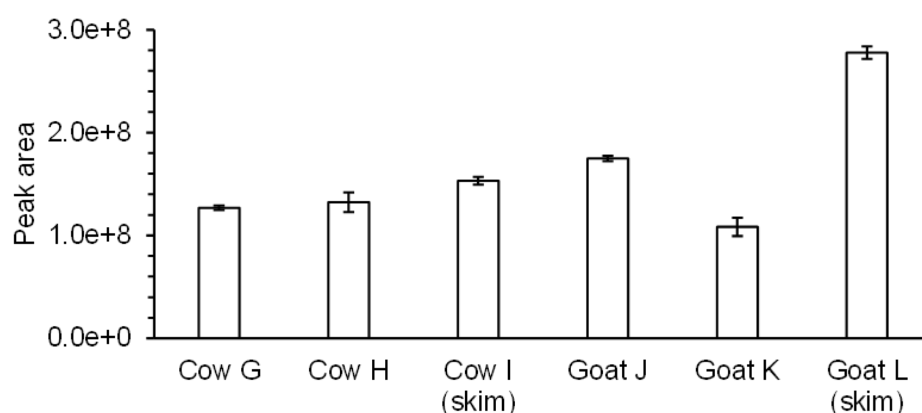

Figure 5. Semi-quantitative comparison of bovine and caprine lactophorins in commercially available milk powders. Results are expressed as normalized LPLSILK peak areas (arbitrary units) per unit weight (grams of milk powder) and shown as mean ± standard deviation, n = 3.

Bovine lactophorin is a heat-stable protein [9] with potential anti-rotavirus [5] and antibacterial properties [7], and its concentration (0.3–0.37 g/L) is high in bovine milk [6,10]. The amino acid sequence of caprine lactophorin shows 89.5% identity with that of bovine lactophorin. Based on the results of this study, the abundance of caprine lactophorin is thought to be nearly the same as that of bovine lactophorin. Lactoferricin derived from caprine lactoferrin in caprine milk possesses antimicrobial activity against a wide range of gram-negative and gram-positive bacteria, fungi, and yeasts [18]. Bovine lactoferricin produced from bovine lactoferrin also shows antibacterial activity [19]. Considering these factors, it is necessary to promote research on the antimicrobial potency of caprine lactophorin.

A limitation of the present study is that lactophorin abundance was evaluated using a semi-quantitative approach based on normalized LPLSILK peak areas without the use of a stable isotope-labeled internal standard. Therefore, absolute concentrations could not be determined and peak areas were normalized to sample volume (milk) or sample weight (milk powder) rather than to total protein content. An additional limitation is the use of a trichloroacetic acid/acetone precipitation protocol for protein extraction. Although this method is effective for global protein precipitation and removal of interfering components, it may not achieve complete recovery of MFGM–associated proteins, such as lactophorin, particularly in milk powders. Accordingly, potential differences in extraction efficiency and overall protein composition across sample types cannot be fully excluded. Taken together, these methodological constraints indicate that the present findings should be interpreted as semi-quantitative, relative comparisons rather than absolute measurements.

4. Conclusions

In the present study, a semi-quantitative LC/MS/MS (MRM) approach was established for the comparative evaluation of lactophorin in bovine and caprine milks and milk powders using the shared proteotypic peptide LPLSILK. Based on normalized peak areas, bovine and caprine milks showed no statistically significant difference in lactophorin-related signal. Because the method relies on relative peak areas without a stable isotope-labeled internal standard, the results should be interpreted as semi-quantitative comparisons rather than absolute concentrations. Nevertheless, the approach provides a practical framework for cross-species comparison of lactophorin abundance across bovine and caprine milks.

Use of AI tools declaration

The author used Copilot in order to improve this article. After using this service, the author reviewed and edited the content as needed and takes full responsibility for the content of the published article.

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Conflict of interest

The author declares no conflict of interest in this paper.

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