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Bovine lactoferrin digested with human gastrointestinal enzymes inhibits replication of human echovirus 5 in cell culture

Camilla B. Furlund[a,b,c], Anja B. Kristoffersen[d], Tove G. Devold[a], Gerd E. Vegarud[a], Christine M. Jonassen[b,c,*]

[a] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1432 Aas, Norway
[b] Department of Medical Microbiology, Akershus University Hospital, 1478 Loerenskog, Norway
[c] Department of Laboratory Services, The Norwegian Veterinary Institute, 0106 Oslo, Norway
[d] Department of Health Surveillance, The Norwegian Veterinary Institute, 0106 Oslo, Norway

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ABSTRACT

Many infant formulas are enriched with lactoferrin (Lf) because of its claimed beneficial effects on health. Native bovine Lf (bLf) is known to inhibit in vitro replication of human enteroviruses, a group of pathogenic viruses that replicate in the gut as their primary infection site. On the basis of a model digestion and human gastrointestinal enzymes, we hypothesized that bLf could retain its antiviral properties against enterovirus in the gastrointestinal tract, either as an intact protein or through bioactive peptide fragments released by digestive enzymes. To test our hypothesis, bLf was digested with human gastric juice and duodenal juice in a 2-step in vitro digestion model. Two gastric pH levels and reduction conditions were used to simulate physiological conditions in adults and infants. The antiviral activity of native bLf and of the digested fractions was studied on echovirus 5 in vitro, using various assay conditions, addressing several mechanisms for replication inhibition. Both native and digested bLf fractions revealed a significant inhibitory effect, when added before or simultaneously with the virus onto the cells. Furthermore, a significant stronger sustained antiviral effect was observed when bLf was fully digested in the gastric phase with fast pH reduction to 2.5, compared with native bLf, suggesting the release of antiviral peptides from bLf during the human digestion process. In conclusion, this study demonstrates that bLf may have a role in the prevention of human gastrointestinal virus infection under physiological conditions and that food containing bLf may protect against infection in vivo.

Abbreviations: bLf, bovine lactoferrin; CPE, cytopathic effect; Ct, cycle threshold; DPBS, Dulbecco phosphate-buffered saline; F, fast reduction in gastric pH; HCMV, human cytomegalovirus; HDJ, human duodenal juice; HGJ, human gastric juice; HPV, human papillomavirus; HS, heparan sulfate; HSV, herpes simplex virus; Lf, lactoferrin; Lfcin, lactoferricin; LfR, lactoferrin receptors; LMW, low molecular weight; MEM, Eagle minimal essential medium; MOI, multiplicity of infection; S, slow reduction in gastric pH; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Si-LfR, small intestinal lactoferrin receptor.

* *Corresponding author*.
E-mail addresses: christine.jonassen@vetinst.no, cjon@ahus.no (C.M. Jonassen).

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1. Introduction

Viral pathogens belonging to several families including *Picornaviridae*, *Caliciviridae*, *Adenoviridae*, *Astroviridae*, and *Reoviridae* can infect the human intestines. Enteric viruses are resistant to the acidic environment in the stomach and are transmitted by the fecal-oral route [1–4]. Most of these viruses are associated with gastroenteritis; however, the enteroviruses, which are small nonenveloped positive stranded RNA viruses that belong to the *Picornaviridae* family, may migrate to secondary organs through the bloodstream after initial replication in the gut. Enteroviruses may cause more severe illness including meningitis, paralysis, myocarditis, and skin diseases [1,3,5].

Infants and young children are more susceptible to infection than adults because they have a less acidic environment in the stomach. Moreover, their digestive systems are not fully developed and they lack prior immunity [6,7]. Several studies have reported that breastfed infants are less susceptible to enterovirus infections; however, this protection decreases with age and reduced doses of milk [8–10]. Milk contains several bioactive proteins, among them lactoferrin (Lf), that have been attributed to a protective role against infections [11].

Lactoferrin is an 80-kd multifunctional glycoprotein that consists of a single polypeptide chain, which is folded into 2 globular lobes (N- and C-lobes) [12–14]. Lactoferrin is produced by mucosal epithelial cells and neutrophils and can be found in mucosal secretions such as tears, saliva, and seminal and vaginal fluids; exocrine secretions such as milk, bile, and pancreatic juice; and small intestinal secretions. Lactoferrin concentrations in milk are species dependent; the concentrations in human milk are higher than that in the milk from ruminants [11,12,14].

Lactoferrin has several biological functions such as regulation of iron homeostasis, bacteriostatic and bactericidal activity, and antifungal and antiviral effects against enveloped and nonenveloped viruses. Therefore, Lf is important in the host defense against microbial infections [11–14].

Specific Lf receptors (LfRs) have been identified on several target cells such as the epithelial cells in the gastrointestinal tract, liver, and brain; monocytes; lymphocytes; and platelets [13,15]. Investigations with enveloped and nonenveloped viruses have demonstrated that Lf exerts an antiviral effect during the early phase of infection, and this effect varies among viral species. Studies on human immunodeficiency virus, herpes simplex virus (HSV), and human cytomegalovirus (HCMV) revealed a direct interaction between Lf and host cells through cell surface receptors [16–18]. In addition to binding to cellular viral receptors, there is evidence that Lf can bind to viral proteins to prevent viral infections, thereby initiating a competition for host cell surface receptors. This finding has been confirmed for enteroviruses [19] and hepatitis C virus [20]. In addition, Lf exerts antiviral activity during an intracellular step that occurs late in viral replication, which has been demonstrated for enteric viruses such as echovirus 6 [21] and simian rotavirus SA-11 [22], and for several other viruses including HSV1 [23] and HCMV [24]. For all these studies, viral inhibition was observed when Lf was added up to 1 hour after the virus adsorption step.

Lactoferrin is partly resistant to digestion in newborns and can be absorbed in an intact form from the gut of infants [13,25,26]. Only a few in vivo studies of Lf digestion have been reported [27,28]. Individual variations in the gastrointestinal content of proteases, amylases, lipases, inhibitors, bile salts, bilirubin, cell mucus, and other minor components significantly influence protein degradation [29–31]. The individual variations that have been observed include differences in the enzymatic activity and volume of the gastrointestinal juices. Gastric pH varies according to age and a fasted or fed stage [30,32–34]. These variations make the simulation of in vivo digestion challenging, and most of the studies on the in vitro digestion of milk proteins have been performed using commercial digestive enzymes [35–38].Differences in the protein and peptide profiles have been indicated when commercial enzymes were compared with human gastrointestinal enzymes in the simulation of in vitro digestion models. These studies demonstrated that human gastrointestinal enzymes are more complex and species related than previously understood [39–41].

Although native bovine Lf (bLf) has been shown to exert antiviral activity on enterovirus in vitro, its antiviral activity at the viral primary replication site in vivo would imply a sustained antiviral activity after the human digestion process. To better understand the value of bLf in human nutrition, we intended, in the present study, to investigate the antiviral effect of bLf after digestion with human gastric (HGJ) and duodenal juices (HDJ). We hypothesized that bLf could retain its antiviral properties in the gastrointestinal tract, either as an intact protein or through bioactive peptide fragments, and thereby protect humans against enteric virus infections. To test our hypothesis, bLf was digested with HGJ and HDJ in a 2-step in vitro model. Two gastric pH levels were used to simulate physiological conditions in adults (pH 2.5) and infants (pH 4.0). In addition, fast and slow pH reductions were used, representing nonbuffering and buffering conditions in the stomach after ingestion of a large meal. Both native bLf and digested bLf were tested for their antiviral activity against echovirus 5, an enterovirus that belongs to subgroup B and is pathogenic to the human gastrointestinal tract. Different antiviral assay conditions were used to determine at which step of viral replication the inhibitory effect of bLf (native and digested) was observed. The antiviral activities of digested bLf after the gastric and duodenal phases were compared with undigested bLf. Our study addresses whether foods rich in bLf may have a role in preventing gastrointestinal virus infections under physiological conditions in humans.

2. Methods and materials

2.1. Cells and virus propagation

Human colorectal adenocarcinoma cells (Caco-2) (ATCC HTB-37) were kindly provided by Dr Erwin Duizer (National Institute of Public Health and Environment, Bilthoven, the Netherlands). The cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in Eagle minimal essential medium (MEM) containing 1.2 g/mL $NaHCO_3$ and supplemented with