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Antiviral Activity of Bovine, Buffalo and Camel Yogurt Against Human Rotavirus in *vitro*

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ABSTRACT

Acute diarrhea, especially in children, is a very common disease with worldwide distribution and with a significant public health impact. Rotaviruses have been recognized as the major agents of diarrhea in infants and young children in developed as well as developing countries. In Egypt, diarrhea is one of the principal causes of death, mainly in the infant population. The antiviral activity of the cell-free supernatants (CFS) containing the metabolites of 6 yogurts and bio-yogurts made from bovine, buffalo and camel fermented milk under anaerobic conditions with bacterial starter, was initially evaluated against human Rotavirus using MTT colormeteric method, In addition to, determine the mode of action of antiviral activity. Then, the effect of pre-heat treatment of milk on antiviral activity of CFS of each yogurt was evaluated. All the CFS of yogurt was exhibited high antiviral activity against Rotavirus. Results obtained demonstrated that all CFS of yogurt can inhibit the replication of rotavirus and able to bind the viral particles to susceptible cells with early phases of rotavirus infection. On the other hand, pre-heat treatment induced losing the antiviral activity of yogurt metabolites with increasing temperature and time of heating. Generally, CFS of the yogurt containing metabolites fermented with probiotic bacteria showed high potential to be used for developing fermented milk-based foods or drugs.

Key words: Rotavirus, Yogurt, Bovine milk, Buffalo milk and Camel Milk.

Introduction

Acute diarrhea, especially in children, is a very common disease with worldwide distribution and with a significant public health impact. Rotaviruses have been recognized as the major agents of diarrhea in infants and young children in developed as well as developing countries [4]. Rotavirus is the most common etiologic agent of health care acquired diarrhea in paediatric patients. Community- and health care–acquired infections have similar temporal distributions; they are caused by the same viral subtypes; and they affect children of the same age groups. All of the health care acquired infections with known viral subtypes occurred while the same subtype was still active in the community, suggesting that health care–acquired infections arise from repeated introduction of the community-acquired rotavirus into the hospital setting. Rotavirus A, which accounts for more than 90% of rotavirus gastroenteritis in humans, is endemic worldwide [13]. Each year rotavirus causes millions of cases of diarrhoea in developing countries, almost 2 million resulting in hospitalisation and an estimated 453,000 resulting in the death of a child younger than five [17,21]. This is about 40 % of all hospital admissions related to diarrhea in children under five worldwide [22].

During the last few years efforts have been made to increase the number of substances with antiviral activity and few belong to the class of nucleoside analogues such as acyclovir and ribavirin [1,7,5]. However, the therapeutic potency of most antiviral agents administered so far is counteracted by their severe side effects in humans [3,11] Therefore, the search for antiviral substances with high efficacy, low toxicity and minor side effects must continue, and there is an urgent need for broad spectrum antiviral drugs that exert inhibitory effects against currently active human viruses.

Although progress has been made during the past few years in developing various live vaccine candidates, up to now, there is no effective one [14]. The only treatment of rotavirus gastroenteritis is the replacement of fluids and electrolytes lost by vomiting and diarrhea [12]. However, rotaviruses cause severe infections in patients undergoing bone marrow transplantation and suffering from a variety of immunodeficiency conditions, there is need for alternative means of protection besides vaccination [6].

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Since the ancient times, natural products have served as a major source of drugs. Interest in natural products as a source of new drugs is growing due to many factors such as; viruses have been resistant to chemical drug therapy or prophylaxis longer than any other form of life and to side effects of synthetic drugs. The growing interest in natural products derived antiviral agents, will significantly expedite the current exploration of the natural environment for compounds with significant pharmacological applications, which will continue to be a promising strategy and new trend for modern medicine [15].

Recently, yogurt was selected as health promoting probiotics and it is a multifunctional foods which exhibits a broad spectrum of antimicrobial properties against bacteria and several viruses include Enterovirus 71, Coxsackie B_3 virus, Coxsackie B_4 virus, Influenza A viruses [9]. This food is captured my attention to study its potential to be an efficient agent to prevent or cure the infection of Rotavirus. The present work was conducted to evaluate the antiviral activity of CFS metabolites of yogurt and bio-yogurt of different sources of milk in *vitro*, on rotavirus infection in MA104 cell line using MTT method. In addition to, determine the mode of action of antiviral activity. Then, the effect of pre-heat treatment of milk on antiviral activity of CFS of each yogurt was evaluated.

Materials and Methods

Viruses, cell lines and reagents:

Rotavirus wa strain was provided by our laboratory at National Research Centre, Egypt and incubated with treated trypsin (10 µg/ml) at 37^{o}C for 30 min and inoculated into MA104 cell line [20]. MA104 (Rhesus monkey epithelial cell line) cell line was maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic–antimycotic solution. Virus titer was determined by CPE inhibition assay and virus stock was stored at -70^{o}C until use. Antibiotic– antimycotic solution, trypsin-EDTA, FBS and DMEM were supplied by Gibco BRL (Grand Island, NY, USA). The tissue culture plates were purchased from Falcon (BD Biosciences, Franklin Lakes, NJ, USA). MTT was purchased Sigma-Chemicals (St Louis, MO, USA) and yogurt starter was provided by Nestlé Co, Egypt. All other chemicals were a reagent grade.

Production of yogurt:

95 ml of full cream bovine, buffalo and camel milks were initially preheated to 41^{o}c and inoculated with 5 gm of bacterial yogurt starter followed by incubated overnight for 1 day at 41^{o}c in incubator. The yogurt formed was kept refrigerated at 4^{o}c [8].

Production of bio-yogurt:

Honey was mixed thoroughly with distilled water in ration 1:10. Then 10 ml of honey solution was mixed with 85 ml of preheated full cream milk at 41^{o}c and 5 gm of bacterial yogurt starter. The mixture was mixed and incubated at 41^{o}c for 24 hr. the pH of the mixture was determined every 30 min by pH meter and the inoculation was terminated at pH 4.5 by placing the mixture in ice-bath for 60 min. Then placed in refrigerator at 4^{o}c [16].

Preparation of the metabolites for measurement antiviral activity and cytotoxicity:

100 ml of each sample of yogurt and bio-yogurt were cooled after 1 day and stored at 4^{o}c. Cell-free supernatants (CSF) of the metabolites were obtained when 10 ml of each samples was diluted with 10 ml of medium to adjust to pH 7.0 value and filtered using 0.22 µm syringe filter (Millipore Corp., Bedford, MA, USA) to remove bacterial and other organisms interrupted growth of cell line. The CFS of yogurt and bio-yogurt were kept frozen at -20^{o}c [9].

Preheat treatment of milk before yogurt production:

6 flasks of 95 ml of full cream bovine, buffalo and camel milks were heated at 60^{o}c, 80^{o}c and 100^{o}c for 5, and 15 min in a thermostatically water bath. The Yogurt was manufactured to the protocol proposed by Elagamy [2] in Triplicate. Milk Samples rapidly cooled to 41^{o}c and inoculated with 5 gm of bacterial yogurt starter and followed by incubated overnight for 1 day at 41^{o}c in incubator. Then, kept a refrigerated at 4^{o}c and yogurt metabolites were extracted as described above.

Assays of cytotoxicity and antiviral activity:

To measure cytotoxicity assay MA104 cells were seeded onto a 96-well culture plate at a concentration of 2 x 10^4 cells per well. Next day, medium was removed and washed with phosphate buffered saline (PBS). The 96-well plates were exposed to CFS (thee wells per CFS) in maintenance medium for 2 days at 37^{o}C, in parallel with the virus-infected cell cultures. For each CFS, four wells were used as controls. After 2 days of incubation, cytotoxicity was evaluated by evidence of morphological change was recorded using the CPE scoring system. The antiviral activities of the CFS against Rotavirus were determined by MTT colormeteric method based on intensity of colour change following the reduction of 3-[4, 5-dimethylthiazol-2-yi]-2, 5-diphenyltetrazplium bromide by mitochondrial enzymes. Subsequently, Confluent 96-well MA104 plats were infected with 100 µl of the diluted virus suspension containing

50% cell culture infective dose ($CCID_{50}$) of the virus stock was added to produce appropriate CPE within 2 days after infection, followed by the addition of 100 µl of CFS. Four wells were used as the virus controls (virus-infected non-CFS-treated cells) and the cell controls (non-infected non-CFS-treated cells). The culture plates were incubated at 37^{o}C in 5% CO_2 for 2 days until appropriate CPE was achieved. The cells were washed with PBS and 20 µl of MTT solution (5 mg/ml stock solution) was added to each well. After 4 hr of incubation at 37^{o}C, the medium was aspirated and formed formazan crystals were dissolved with 200 µl of acidified isopropanol. Absorbance of formazan solutions were measured at λ540 nm with 620 nm as a reference wavelength using a multiple-well plate reader [20]. The results were expressed as a percent achieved by tested metabolites in the virus-infected cells was calculated using the following formula [(O.Dtv-O.Dcv) / (O.Dcd-ODcv)] x 100% where O.Dtv, O.Dcv and O.Dcd are the optical density of tested compound, the optical density of virus control and the optical density of cell control respectively, antiviral activity was presented as % of control.

The mode of action of antiviral activity against Rotavirus:

Viral adsorption:

Confluent monolayer of MA104 cells were grown in 96-well plates and inoculated with 100 µl of CFS of metabolites for 90 min at 37^{o}C in 5% CO_2. The medium was aspirated and 100 µl of Rotavirus was inoculated and incubated for 90 min at 37^{o}C in 5% CO_2. Then virus suspension was removed and 100 µl of maintenance medium was added. Then further incubation time was done for 2 days at 37^{o}C. The cells were washed with PBS and 20 µl of MTT solution was added. After 4 hr of incubation at 37^{o}C, the medium was aspirated and formed formazan crystals were dissolved with 200 µl of acidified isopropanol. Absorbance of formazan solutions were measured and expressed as described above.

Viral attachment:

100 µl of Rotavirus was inoculated with equal volume of CFS of metabolites for 90 min at 37^{o}C in 5% CO_2. 100 µl of mixed suspension were added to Confluent monolayer of MA104 which was grown in 96-well plates. After 90 min of incubation, mixed suspension was replaced with 100 µl of maintenance medium. The plates were incubated for 2 days. Then cells were washed with PBS and 20 µl of MTT solution was added. After 4 hr of incubation at 37^{o}C, the medium was aspirated and formed formazan crystals were dissolved with 200 µl of acidified isopropanol. Absorbance of formazan solutions were measured and expressed as described above.

Viral replication:

This experiment was carried out as stated above sections with the following differences: Confluent monolayers of MA104 cell line was grown in 96-well plates and treated with 100 µl of Rotavirus for 90 min. Cells were washed, overlaid with 100 µl of CFS of metabolites, and incubated for 90 min at 37^{o}C. Then 100 µl of maintenance medium was added. The plates were incubated for 2 days. Viral evaluation following the procedures described above.

Results:

Antiviral activity and cytotoxicity of CFS of yogurts against Rotavirus:

The CFS of each yogurt and bio-yogurt were tested for antiviral activity against Rotavirus. The CFS of bovine yogurt, camel bio-yogurt, camel yogurt and buffalo yogurt exhibited significant strong inhibitory effects against Rotavirus of 91.5 %, 90.3 % 88.90 % and 88 % respectively, and those of buffalo bio-yogurt and bovine bio-yogurt exhibited moderate antiviral activity of 86.1% and 82 % respectively (Table 1). The CFS of each yogurt and bio-yogurt did not exhibit cytotoxicity in MA104 cells at tested concentration (data not shown).

Mode of action of antiviral activity of CFS yogurts against Rotavirus:

All tested metabolite were exhibited a weak antiviral activity less than 50 % during virus adsorption step except CFS of camel bio-yogurt, bovine yogurt and camel yogurt of 61.3 %, 61.2 % and 57.2 % respectively, and during virus multiplication step, all CFS exhibited moderate antiviral activity up to 79.9 %, 75%, 72.4% and 71.5 of CFS of buffalo bio-yogurt, bovine yogurt, bovine bio-yogurt and camel bio-yogurt respectively. But during viral attachment step, the antiviral activity was increased more than previous two steps; it is sometimes up to 86.3 % and 79 % of bovine and camel yogurt metabolites (Table 1).

Effects of pre-heat treatment of various milk on antiviral activity of CFS yogurts against Rotavirus:

Results showed that the efficiency of antiviral activity of bovine, buffalo and camel yogurt metabolites were changed during various degrees of heat treatment of milk against Rotavirus in comparison with antiviral activity control which were 91.5%, 88% and 88.9% before heat treatment respectively, and became 60%, 53%, 87.5% after heat treatment at 60^{o}C for 5 min, 47%, 28%, 76% at 60^{o}C for 15 min, and 26%, 11%, 63% at 80^{o}C for 5 min respectively. But during heat treatment with 80^{o}C for 15 min, no antiviral activity was appeared

except CFS of camel milk yogurt which was 30%. And all CFS of yogurts have no antiviral activity during heat treatment at 100°C (Table 2).

Table 1: Antiviral activity and mode of action of yogurt metabolites against Rotavirus

CFS of Yogurt	% Inhibitory Activity (mean ± S.D) against Rotavirus			
	Antiviral Activity	Virus Adsorption	Virus Multiplication	Virus Attachment
Bovine Yogurt	91.5 ± 2.30	61.2 ± 2.70	75.0 ± 2.9	86.3 ± 1.80
Buffalo Yogurt	88.0 ± 2.90	31.0 ± 0.90	53.4 ± 1.3	32.3 ± 1.77
Camel Yogurt	88.9 ± 1.94	57.2 ± 0.71	66.6 ± 2.0	79.0 ± 2.20
Bovine bio-yogurt	82.0 ± 2.70	44.2 ± 1.10	72.4 ± 1.5	63.1 ± 1.88
Buffalo bio-yogurt	86.1 ± 0.80	49.0 ± 0.70	79.9 ± 1.3	69.3 ± 1.00
Camel bio-yogurt	90.3 ± 3.50	61.3 ± 2.90	71.5 ± 2.4	70.0 ± 2.60
Values represent the means of three independent experiments. Antiviral activity was presented as % of control. S.D Standard Deviation				

Table 2: Effects of pre-heat treatment of milk on antiviral activity of CFS yogurt against Rotavirus

CFS of Yogurt	% Inhibitory Activity against Rotavirus						
	Antiviral Activity Control	60°C		80°C		100°C	
		5 min	15 min	5 min	15 min	5 min	15 min
Bovine Yogurt	91.5 ± 2.30	60 ± 2.9	47 ± 3.4	26 ± 3.5	NA	NA	NA
Buffalo Yogurt	88.0 ± 2.90	53 ± 3.0	28 ± 4.1	11 ± 4.3	NA	NA	NA
Camel Yogurt	88.9 ± 1.94	87.5 ± 2	76 ± 2.6	63 ± 2.6	30 ±3.5	NA	NA
Values represent the means of three independent experiments. Antiviral activity was presented as % of control. S.D Standard Deviation & NA No Antiviral Activity							

Discussion:

This study examined the antiviral activity of CFS of the metabolites of various yogurts made from bovine, buffalo and camel milk in vitro, on rotavirus infection in MA104 cell line using MTT colormeteric method. The obtained results demonstrated an inhibition of Rotavirus as a result of treatment with tested CFS, which were exhibited a significant inhibitory effects against Rotavirus in comparison with a control. Also it is able to inhibit Rotavirus during various steps of infection as adsorption, attachment and multiplication with different percentages. The CFS of bovine yogurt, camel bio-yogurt, camel yogurt and buffalo yogurt exhibited a strong inhibitory effect against Rotavirus of 91.5 %, 90.3 % 88.90 % and 88 % respectively, and those of buffalo bio-yogurt and bovine bio-yogurt exhibited moderate antiviral activity of 86.1% and 82 % respectively.

A previous study found that lactoferrin is active against a simian rotavirus SA11 in *vitro* and the apo-LF was as potent against this rotavirus as the iron-saturated holo-LF form [19]. Also, Superti *et al*., [18] confirmed that lactoferrin prevents rotavirus infection mainly through binding to viral particles; and milk component may interfere with an intracellular step of virus infection since specific receptors. However, a few studies looking into antiviral activity of low molecular materials as well as metabolites of yogurt have been published. Our results showed similar results to previous findings by Hwa-Jung *et al*., [10], who's demonstrated that CFS of bacterial yogurt metabolites have strong antiviral activity against RNA viruses such as Coxsackie B_3, Coxsackie B_4, Echoviruses and Influenza A viruses.

Based on the results were obtained from the mode of action experiment's, are confirmed on the antiviral activity of CFS of the metabolites of yogurts at different steps of viral infection and also, the inhibition percentage less than 50% during viral Adsorption, attachment and multiplication steps may be due to that these CFS of the yogurt metabolites plays more than a role because the CFS of yogurt metabolites were incubated with the cells for various periods of time and at different intervals before or after virus binding.

The preservation of raw milk can be achieved by heat treatments such as pasteurization, boiling or sterilization processes. These treatments have direct influences on the nutritional, biological and functional properties of milk. No data are present in the literature on the thermal effect on antiviral activity of milk or its product. Our results suggested that heat-induced losing the antiviral activity of yogurt metabolites with increasing temperature and time of heating as a present in Table 2. All CFS of yogurt metabolites extracted from heating milk at 100°C were completely loss their antiviral activity against Rotavirus and also, all CFS of yogurt metabolites extracted from heating milk at 80°C for 15 min were completely loss their antiviral activity except CFS of camel yogurt metabolites. And this suggests that CFS of camel milk as a basic component of yogurt metabolite was markedly more heat resistant than CFS of Cow and buffalo milks as a basic component of yogurt metabolites and preserved its antiviral activity during various heat treatments.

In conclusion:

Fermented food can be considered as a good food of high nutritive and therapeutic applications. Yogurt fermented with bacterial starter may possess strong antiviral materials against Rotavirus. Further

studies are necessary to isolate specific antiviral compounds and further research is needed to investigate the effects of CFS of yogurt metabolites against Rotavirus and other viruses in *vitro* and in *vivo* for the development of effective antiviral drugs.

References

1. De Clercq, E., 2004. Antiviral Drugs in Current Use. J. Clin. Virol., 30(2): 115-133.
2. Elagamy, E., 2000. Effect of heat treatment on camel milk proteins with respect to antimicrobial factors: a comparison with cows' and buffalo milk proteins. Food Chem., 68: 227-232.
3. Englund, J.A., M.E. Zimmerman, E.M. Swierkosz, J.L. Gooidman, D.R. Scholl, H.H. Balfour, 1990. Hepes Simplex Virus Resistant to Acyclovir. Astudy in Tertiary Care Center. Ann. Intern. Med., 112(6): 416-0422.
4. Estes, M.K., and A.Z. Kapikian, 2007. Rotaviruses and their replication. In Fields Virology, 5th ed. Pp: 1917–1974. Edited by Fields B.N., D.M., Knipe , P.M., Howley, D.E., Griffin, R.A., Lamb, M.A., Martin, B., Roizman, S., Straus. E.Philadelphia, PA: Lippincott, Williams & Wilkins.
5. Fyfe J.A., P.M. Keller, P.A. Furman, R.L. Miller, G.B. Elion, 1978. Thymidine Kinase from Herpes Simplex Virus Phosphorylates the New Antiviral Compound, 9-(2-Hydroxyethoxyl) guanine. J. Biol. Chem., 253: 8721- 8727.
6. Goncalves, J., R. lopes, D. Oliveira and M. Wigg, 2005. In vitro anti-rotavirus activity of some medicinal plants used in Brazil against diarrhea. J. of Ethanopharma., 99: 403-407.
7. Hruska, J.F., J.M. Bemstein, R.G. Douglas, B.V. Hall, 1980. Effects of Ribavirin on respiratory Syncytial Virus in *Vitro*. Antimicrob. Agents Chemother., 17(5): 770-775.
8. Harun-ur-Rashid, M.d., K. Togo, M. Ureda, and T. Miyamoto, 2007. Probiotic characteristics of lactic acid bacteria isolated from traditional fermented milk in Bangladesh. Pak. J. Nut., 6: 647-652.
9. Hwa-Jung Choi, Jae-Hyoung Song, Young-Joon Ahn, Seung-Hwa Baek, Dur-Han Kwon, 2009. Antiviral activities of cell-free supernatants of yogurts metabolites against some RNA viruses. European Food Research and Technology, 228(6): 945- 950.
10. Hwa-Jung Choi, Jae-Hyoung Song, Young-Joon Ahn, Seung-Hwa Baek, Dur-Han Kwon, 2010. Antiviral activity of yogurt against Enterovirus 71 in Vero Cells. Food Sci. Biotech., 19(2): 289-295.
11. Janai, H.K., M.I. Marks, M.Zaleska, H.R. Stutman, 1990. Ribavirin: adverse drug reactions, 1986 to 1988. Pediatr. Infect. Dis J., 9(3): 209-211.
12. Kapikian, A.Z., Y. Hoshino, R.M. Chanock, 2001. Rotaviruses. In: Fields, B.N., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, I.L., Monath, T.P., Roizman, B., Straus, S.E. (Eds.), Virology, fourth ed. Lippincott/ Williams and Wilkins, Philadelphia, PA, pp: 1787-1825.
13. Leung, A.K., J.D. Kellner, H.D. Davies, 2005. "Rotavirus gastroenteritis". Adv. Ther., 22(5): 476-
87. doi:10.1007/BF02849868. PMID 16418157.
14. MMWR, 1999. Intussusceptions among Recipients of Rotavirus Vaccine, United States, 48(27): 577-581.
15. Nagai, T., S. Makino, S. Ikegami, H. Itoh, H. Yamada, 2011. Effects of oral administration of yogurt fermented with Lactobacillus delbrueckii ssp. Bulgaricus OLL1073R-1 and its exopolysaccharides against influenza virus infection in mice. International immunopharmacology, 11: 2246-2250.
16. Shah, N.P., 2003a. Yogurt: the product and its manufacture. In Encyclopedia of Food .Science and Nutrition, Vol. 10, 2nd ed. Caballero B, Trugo L C and Finglas P M, eds.
17. Simpson, E., S. Wittet, J. Bonilla, k. Gamazina, L. Cooley, J.L. Winkler, 2007. "Use of formative research in developing a knowledge translation approach to rotavirus vaccine introduction in developing countries". BMC Public Health 7: 281. doi:10.1186/1471-2458-7-281. PMC 2173895.PMID 17919334.
18. Superti, F., R. Siciliano, B. Rega, F. Giansanti, P. Valenti and G. Antonini, 2001. Involvement of bovine lactoferrin metal saturation, sialic acid and protein fragments in the inhibition of rotavirus infection. Biochimica ET Biophysica Acta, 1528: 107-115.
19. Superti, F., M. Ammendolia, P. Valenti, L. Seganti, 1997. Antirotaviral activity of milk proteins: lactoferrin prevents rotavirus infection in the enterocyte-like cell line HT-29. Med. Microb. Immunol. 186: 83-91.
20. Takahashi, K, M. Matsuda, K. Ohashi, K. Taniguchi, O. Nakagomi, Y. Abe, S. Mori, N. Sato, K. Okutani, S. Shigeta, 2001. Analysis of anti-rotavirus activity of extract from Stevia rebaudiana. Antiviral Res., 49(1): 15-24.

21. Tate J.E., A.H. Burton, C. Boschi-Pinto, A.D. Steele, J. Duque, U.D. Parashar, 2012. Estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis". Lancet Infect. Dis., 12(2): 136 - 141. doi:10.1016/S1473-3099(11)70253-5. PMID 22030330
22. UNICEF/WHO, 2009. Diarrhoea: Why children are still dying and what can be done. Retrieved 23 May 2010.