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The Biological Utilization of Gaddi Sheep's Milk Oligosaccharides

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ABSTRACT: Carbohydrate is a natural constituent of all living beings like: bacteria, fungi, plants and placental mammals' milk, in which oligosaccharide are the main constituents. The milk is a rich source of oligosaccharides with different novel oligosaccharides depending on the nature of the origin of mammals the milk belongs. One of the most important roles for milk is to support the immune system of a newborn, and provide bioactive molecules that allow the gastrointestinal tract of the new born to mature, this maturation play important role in development of a healthy balance of microflora. Goat milk oligosaccharides have anti-inflammatory effects. It is valuable for intestinal protection and fix after the harm brought about by DSS (Dextron sodium sulphate)-induced colitis and their implication in human intestinal inflammation. Bison milk oligosaccharides have resistivity against parasitic diseases. Donkey milk oligosaccharides stimulate non-specific and specific immunological resistance. The cows' milk oligosaccharides decrease the addition of enterotoxic Escherichia coli strains of the calf. Keeping all biological activity of different milk in mind, Gaddi sheep's milk was collected and processed by Modified method of Kobata and Ginsburg and afterward purified by Sephadex G-25 Gel column. The homogeneity was affirmed by reverse phase high performance chromatography. The acetylation of oligosaccharides mixture followed by the silica gel chromatography led to isolation of novel oligosaccharides. The contagious action of Gaddi sheep's milk oligosaccharides was explored against three types of Trichoderma, to be specific T. polysporum, T. flavofuscum and T. longibrachiatum, based on their importance as human pathogens. All samples of milk oligosaccharide exhibited fungal activity against these fungal strains.

KEYWORD: Milk Oligosaccharides, Trichoderma, Microbial Activity, NMR.

I. INTRODUCTION

Oligosaccharides are innate elemental part of all bacteria, fungi, plants and placental mammals' milk. The milk is abundances in oligosaccharides it can provide number of novel oligosaccharides based on the nature of different origin in to which mammals the milk belongs. In addition to lactose, milk contains numerous glycoproteins and a variety of free oligosaccharides. Oligosaccharides with potential physiological adhesiveness might be found in animal milks. The Elephant milk oligosaccharide fraction contained a high ratio of sialyl oligosaccharides this may be significant with respect to the formation of brain components, such as gangliosides of the suckling calves. N-acetylneuraminlactose sulphate will be allowed to play an important role in the nutrition of the rat pups, which is the regnant oligosaccharide in the dog milk. Buffalo milk oligosaccharides have capacity to encourage undefined immunological resistance of the host against parasitic infections. Donkey milk oligosaccharides have ability to triggered indefinite and definite immunological resistance. Goat milk oligosaccharides allocate a crucial role in intestinal protection and repair when an injury caused by DSS (Dextron sodium sulphate)-induced colitis and their relevance in human intestinal inflammation. Goat milk oligosaccharides have anti-inflammatory effects in rats with trinitrobenzenesulfonic (T) acid induced colitis and will be helpful in the management of inflammatory bowel disease. The cows' milk oligosaccharides scale back the adhesion of enterotoxic Escherichia coli strains of the calf. Human milk oligosaccharides are fruitful of postnatal development of the immune system, the protective impact of human milk against viral and bacterial infections, and therefore the improvement of the bioavailability of minerals. The anti-infective impact of human

milk has been partly attributed to the high quality of free oligosaccharides in addition as glycoconjugates, and additionally resistant against digestion. The Elephant milk oligosaccharide fraction contained a high quatitative relation of sialyl oligosaccharides; this could be important with relation to the formation of brain elements like gangliosides of the suckling calves. N-acetylneuraminlactose sulfate could play an essential role within the nutrition of the rat pups, that is the assertive oligosaccharide within the dog milk. Buffalo milk oligosaccharides have resistivity contrary to parasitic infections. Donkey milk oligosaccharides have potentiality to stimulate non-specific and specific immunological resistance. Remember these things i.e. the bioactive properties of all different mammals' milk oligosaccharides, Gaddi sheep's milk was taken in bulk and processed by altered method of Kobata and Ginsburg and then it was purified by Sephadex G-25 Gel column. The correlation is further confirmed by reverse phase high performance chromatography and supported by thin layer chromatography. Acetylation of oligosaccharides mixture followed by the silica gel chromatography direct to separation of number of novel oligosaccharides. These crude oligosaccharide mixtures are farther test for microbial activity.

II. MATERIAL AND METHODS

2.1. GENERAL PROCEDURE

2.11. Optical rotations were delibrated with the help of a PERKIN-ELMER 241 automatic polarimeter in 1cm tube. NMR [1H and 13C] spectra of oligosaccharides were noted in D_2O and the spectra of acetylated oligosaccharides were transcripted in $CDCl_3$ at 25^0C by Bruker AM 300 FT NMR spectrometer. The electrospray mass spectra were noted by MICROMASS QUATTRO II triple quadrupole mass spectrometer. The sample (dissolved in suitable solvents such as methanol/acetonitrile/water) was imported into the ESI source through a syringe pump at the rate 5µl per min.

The voltage of ESI capillary was set at 3.5 KV with cone voltage at 40 V. The spectra were observed in 6s scans with the print outs are averaged spectra of 6-8 scans. An elemental analyzer CARLO-ELBA 1108 recorded analysis of C, H and N. The sugars were visualized on TLC with 50% aqueous H_2SO_4 reagent and on Paper Chromatography with acetyl acetone and p-dimethyl amino benzaldehyde reagents. Silica gel G (SRL) and CC silica gel (SRL, 60-120 mesh) are used as absorbent for TLC. Whatman No.1 filter paper is used for PC with solvent system ethyl acetate-pyridine (2:1) saturated with H_2O. For gel permeation chromatography Sephadex G –25 (PHARMACIA) was used. The compound was freeze drying with the help of CT 60e (HETO) lyophylizer and further centrifuged and cooled by a cooling centrifuged Remi instruments C-23 JJRCI 763. Homogeneity of the compounds was checked by reverse phase HPLC system was used equipped with Perkin Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. Authoritative samples of glucosamine, galactosamine, galactose and glucose were purchased from Aldrich Chemicals.

2.12. Separation of Oligosaccharides Mixture by Kobata and Ginsburg Method- 10L of Gaddi Sheep's milk was taken from a sheep which belongs from northern himalayan region and was stored at -20^0C. The milk was processed by the method of Kobata and Ginsburg. Milk was centrifuged for 15-20 min with 5000 rpm at -4^0C. Cold glass wool column was used to separate the solidified lipid layer. Ethanol was added to the clear filtrate to a final concentration of 68% and the resulting solution was left for 10-12hours at 0^0C. The white precipitate accessed which was rich of lactose and protein was further eliminated by centrifugation and washed twice with 68% ethanol at 0^0C. The supernatant and washings were united and filtered through a micro filter (0.24 mm) (for removal of remaining lactose) and lyophilized affording crude oligosaccharide mixture (162 g). This lyophilized material which primarily consists of mixture of oligosaccharides, was further purified by fractionating it on Sephadex G-25 chromatography with use of glass triple distilled H_2O as fluent at a velocity of 5 min/mm. Each fraction was analyzes by phenol sulphuric acid reagent to check the presence of neutral sugar. The correlation of oligosaccharides in Gaddi sheep's milk was further confirmed by reverse phase high performance liquid chromatography [HPLC] which also supported by thin layer chromatography [TLC], NMR and Mass spectroscopy.

2.2. Fungal Test: Microbial activity of the milk extracts was assessed against various fungi. A suspension of the milk oligosaccharide (of approximately 1–2 mg) is prepared and then spread evenly onto an appropriate agar.

2.21. Preparation of Potato Dextrose Agar Medium-

(i) Composition* Potato Dextrose Agar:

Ingredients	**Gms/Litre**

Potato, infusion from	200.0
Dextrose	20.0
Agar	15
Final pH (at 25^0C)	5.6 ± 0.2

*Formula adjusted, standardized to achievement of parameters

(ii) Preparation: Suspend 39grams in 1000ml distilled water. Mixture heat till boiling to dissolve the medium completely. Suspension was sterilize by autoclaving at 15lbs pressure with 121^0C temperatue for 15minutes. Mix well before dispensing. When pH required 3.5, acidify the medium with sterile 10% tartaric acid/lactic acid. 100ml of acid required to sterile, cooled medium is approximately 1ml. Medium is not heated after addition of acid. Potato dextrose agar is cream to yellow homogeneous free flowing powder with light amber color clear to slightly opalescent gel form in petridish. Potato dextrose agar is endorsed by APHA and F.D.A. for plate counts of yeasts and moulds in the examination of food and dairy products. Potato infusion and dextrose inhanced the luxuriant fungal growth. Adjusting the pH of medium by tartaric acid to 3.5 inhibits the bacterial growth. Heating the medium next to acidification should be avoided because it may hydrolyze the agar, which can render the agar unable to solidify.

2.22. Preparation of CZAPEK DOX Agar Medium-

(i) Composition for CZAPEK DOX Agar:

Ingredients	**Gms/Litre**
Sucrose	30.0
Sodium Nitrate	2.0
Dipotassium Phosphate	1.0
Magnesium Sulphate	0.50
Potasium Chloride	0.50
Ferrous Sulphate	0.10
Agar	15
Final pH (at 25^0C)	7.3 ± 0.2

(ii) Preparation: Suspend 49 grams of Czapek Dox Agar in 1000ls of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 15lbs pressure at 121^0C for 15 minutes.

CZAPEK DOX Agar is a semi-synthetic medium which contains sodium nitrate as the one and only source of nitrogen. It is used for the agrology of fungi. The medium has favorable buffering action by cause of the presence of different salts. It is a creamish white, uniform, free flowing powder with light yellow colored, clear to more or less opalescent gel. CZAPEK DOX Agar reinforced bountiful cultivation of almost all saprophytic fungi with specific mycelia and conidia formation.

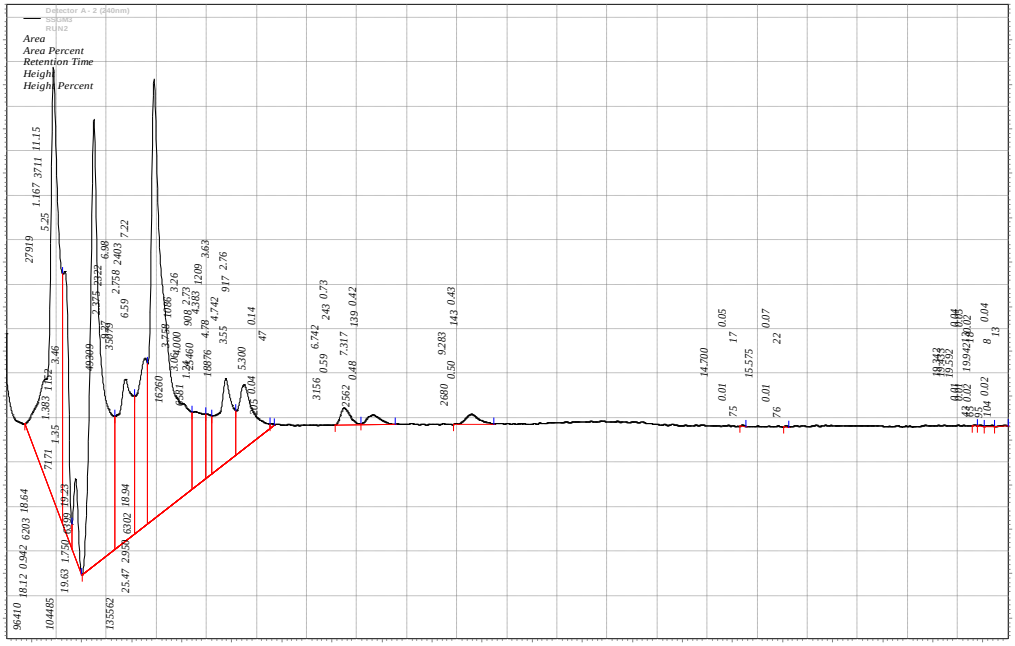
2.23. Potato dextrose agar medium and CZAPEK DOX agar medium was used for Trichodema species viz; T. polysporum, T. flavofuscum and T. longibrachiatum, spread over in a petri dish. pH adjusted to 5.40-5.80 for potato dextrose agar medium at 20-25 °C and at 7.3 ± 0.2 for CZAPEK DOX agar medium. The final concentrations of Gaddi sheep's milk oligosaccharides were 25,15, 5, 1 and 0 μg mL^{-1}. Pure culture of the fungi was taken and 0.1 ml of both cultures was spread on the surface of media plate with the help of sterile swab. After the plates were allowed to dry, sterile paper disks containing Gaddi sheep's milk oligosaccharide and double-distilled H_2O were placed on the agar plate surface. Then, the plates were incubated at 28 °C for 5-7days for Trichoderma species.

III. RESULT AND DISCUSSION

3.1. Confirmation of Homogeneity of Gaddi's Milk Oligosaccharide by Reverse Phase HPLC-

Mixtures of oligosaccharides were quantitatively analyzed by reverse phase HPLC. The HPLC system was bedecked with Perkin-Elmer 250 solvent conveying system, 235 diode array detector and G.P. 100 printer plotters. Cyano column used were binary gradient. Number of eluents were detected at 240 nm. Twenty-one peaks were observed in the sample at the varied retention times from 00.942min. to 19.942min. the increasing order of retention time are: 19.942 min. (R_1),19.592 min. (R_2),19.435 min. (R_3),19.342 min. (R_4),15.575 min. (R_5), 14.700 min. (R_6),09.283 min. (R_7),07.317 min. (R_8),06.742 min. (R_9),05.300 min. (R_{10}),04.742 min. (R_{11}),04.383 min. (R_{12}),04.000 min. (R_{13}),03.758 min. (R_{14}),02.950 min. (R_{15}),02.758 min. (R_{16}),02.375 min. (R_{17}),01.750 min. (R_{18}),01.383 min. (R_{19}),01.167 min. (R_{20}) & 00.942 min. (R_{21}).

90% TDW: 10% ACN in water 240 nm—

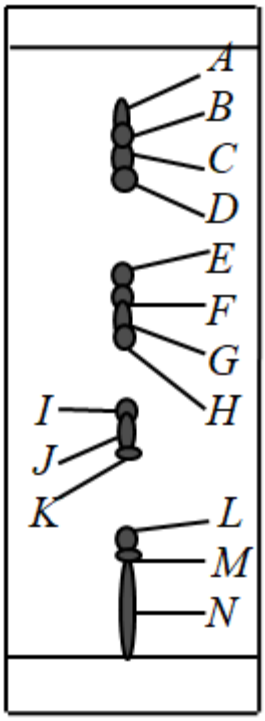


HPLC Table of Crude Gaddi Sheep's Milk Oligosaccharides -

PK	Retention time	Area	Area %	Height	Height%
1	19.942	0013	00.04	000104	00.02
2	19.592	0008	00.02	000095	00.02
3	19.435	0018	00.05	000065	00.01
4	19.342	0013	00.01	000043	00.01
5	15.575	0022	00.07	000076	00.01
6	14.700	0017	00.05	000075	00.01
7	09.283	0143	00.43	002680	00.50
8	07.317	0139	00.42	002562	00.48
9	06.742	0243	00.73	003156	00.59
10	05.300	0047	00.14	000205	00.04
11	04.742	0917	02.76	018876	03.55
12	04.383	1209	03.63	025460	04.78
13	04.000	0908	02.73	006581	01.24
14	03.758	1086	03.26	016260	03.06

15	02.950	6302	18.94	135562	25.47
16	02.758	2403	07.22	035079	06.59
17	02.375	2322	06.98	049309	09.27
18	01.750	6399	19.23	104485	19.63
19	01.383	1152	03.46	007171	01.35
20	01.167	3711	11.15	027919	05.25
21	00.942	6204	18.64	096410	18.12

3.2. Method of Acetylation of Oligosaccharide Mixture-- 22gm of coarse oligosaccharide mixture was acetylated with pyridine (20ml) and acetic anhydride (20ml) at 600 C and solution was stirred for 10-12hours. The mixture was evaporated beneath reduced pressure and the viscous residue was held in CHCl3 (500ml) and washed in with ice-cold water. The organic overlay was dried on anhydrous Na2SO4, filtered and evaporated to dryness give in to the acetylated mixture (26.50gm). By acetylation the free sugars are converted into their nonpolar acetyl derivatives which were resolved admirably on TLC plates, giving ten spots on TLC i.e. A, B, C, D, E, F, G, H, I, J, K, L, M & N of which eleven compounds were finally separated by column chromatography over silica gel using hexane:chloroform, chloroform and CHCl3:MeOH as eluents. Thin Layer Chromatography (TLC) of acetylated Gaddi Sheep's milk oligosaccharides.



$CHCl_3$: MeOH (95:5)

Inhibition was observed by any of the milk extract against the above said fungi. Pre-known references for comparison in fungal tests are used. All experimental procedures were performed in triplicates.

3.3. Fungal Test

Fungi, viz: Trichoderma, to be specific T. polysporum, T. flavofuscum and T. longibrachiatum, are used. Fungal activity examination remains an area of intense interest. Susceptivity testing can be used for drug discovery and epidemiology. Number of reports is available showing efficiency of different milk as antifungal agents. Gaddi sheep's milk enhanced the growth of Trichoderma species.

Trichoderma are developed as biocontrol porter for fungal diseases of plants. Trichoderma polysporum produced Cyclosporine A (CsA), a calcineurin resister, it is an immunosuppressant prescribed in organ transplants to stop rejection. Trichoderma longibrachiatum, the common house mould, produces small mephitic peptides enclose of amino acids absent in common proteins, like alpha-aminoisobutyric acid, known as

trilongins. Trilongins are extremely coercive to heat and antimicrobials making primary interference the sole management option. Trichoderma polysporum was shown to have biocontrol activity outside of caves against a variety of other fungi including Armilaria gallica, Fomus annosus, and Ceratocystis paradoxa. Trichoderma flavofuscum have pathogenic potential although they are not commonly thought of as a disease causing agent in humans and animals. Trichoderma is a potent biocontrol agent and used extensively for soil born diseases and plant growth promotor to increase the plant's ability to resist drought. Trichoderma strains are known for increses resistance in plants. Some species of Trichoderma by secretion of specific compounds induce ethylene formulation, hypersensitive reposte and other defensive reflection in plant cultivars. Trichoderma strains are remarable in the bioremediation of soil that are unkempt by pesticides and herbicides. They have potentiallity to degrade a huge range of disinfectant: organochlorines, organophosphates and carbonates. Gaddi sheep's milk oligosaccharides at the concentrations of 1, 5, 10, 15 and 20mg/ml. In each extract have promising fungal activity against T. polysporum, T. flavofuscum and T. longibrachiatum.

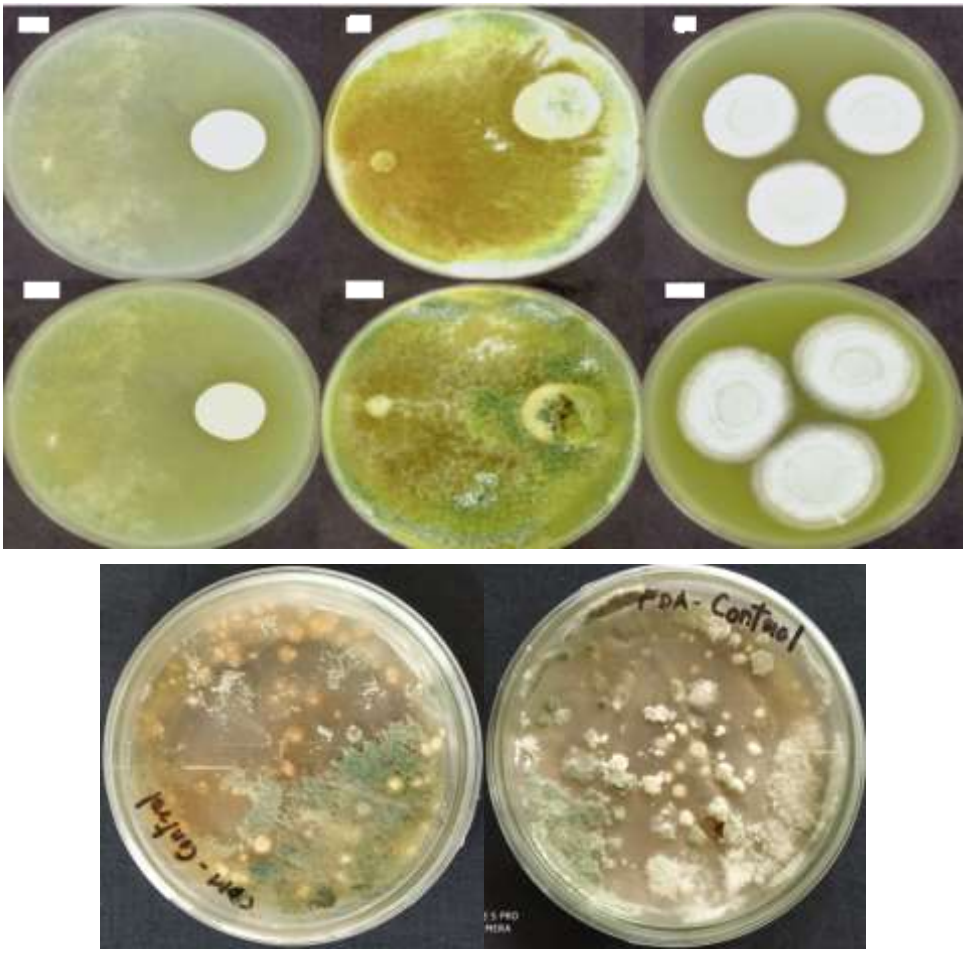


Figure: Inhibition zones of T. polysporum, T. flavofuscum and T. longibrachiatum growth on Gaddi sheep's milk oligosaccharide the peripheral wells contained extract concentrations (1, 5 10, 15 and 20mg/ml).

We found three fungal strains: T. polysporum, T. flavofuscum and T. longibrachiatum was significantly sensitive to Gaddi sheep's milk oligosaccharides. The growth of these strains was enhanced by nearly 68%, 55% and 60%, respectively after 24 h of culture.

IV. CONCLUSION

Finally, based on results acquired from the above investigation, the microbial action of Gaddi sheep's milk against different species of Trichoderma which retains natural contamination, mostly oil based, enhanced their growth. It might be considered as important natural contamination controller with Trichoderma as in type of sprayer, compost or to grow fungi either in indoor and outdoor and so on to made clean and green environment.

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