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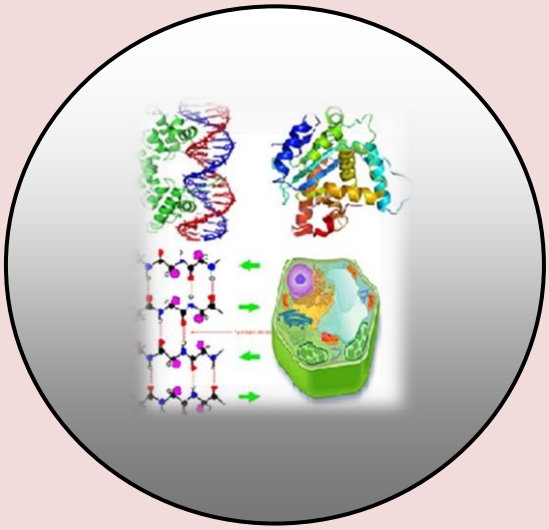

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RESEARCH PAPER

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Structures of Biologically Active Milk Oligosaccharides Isolated from Various Indigenous Cow Species

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ABSTRACT

In Ancient Indian literature in general and in Ancient medicinal system like Ayurveda and Charak Samhita particularly, cow and its milk is glorified because of its medicinal values. It may be provided to the newly born as their food and younger and older persons for their growth and development. Cow's milk develops the immune system, develops brain and bones. It has been defined as Amrita in Ancient literature and has immunomodulatory, anti-inflammatory and antioxidant properties. It also increases the lactation process in the feeding women. In Ancient times, there was limited species of cow which gave minimal milk. Moreover, in present time, with the variety of cows like Jarsi, Frazier, Sahiwal, Tharparkar, Kankrej and Rathi cow, the amount of milk has been increased. The cow's milk is comprised of proteins, fats, carbohydrates, vitamins and minerals. The carbohydrate content is comprised mainly of lactose and the oligosaccharides. The biological properties described above are mainly due to these oligosaccharides which are long and branched chain of Glc, Gal, GlcNAc and GalNAc etc. In this article, we have described the method of isolation of milk oligosaccharides from various species of cow's milk. Further, we have described the stereoscopic structures of these oligosaccharides, describing their sequence, configuration and conformation of 25 novel oligosaccharides which has been isolated in our laboratory and their structure elucidation has been performed with the help of physico chemical techniques like ^{1}H NMR, ^{13}C NMR, 2D NMR viz. HSQC, TOCSY, COSY, HMBC and Mass spectrometry.

Keywords: Milk, Oligosaccharides, Cow, NMR and Structure elucidation.

INTRODUCTION

Indian cow is virtuous not just with reference of being auspicious, it hold a prominent place in Vedic literatures because its milk contribute a lot for human development, since its birth. The biological importance of cow's milk is well defined in the Indian medicinal systems like Chark Samghita and Ayurveda where it is described as amrita the pious drink it is clearly placed that cow's milk is a replacement to mother's milk and is responsible for the development of the immune system, brain and skeletal system of the neonate. Since the cow milk is consist of protein, fat, and carbohydrate in the form of lactose and oligosaccharides. Recent researches on oligosaccharide content of the cow milk of various indigenous breeds have shown varying biological activities i.e. brain development (Uemura et al., 2006), immunomodulatory (Cross and Gill, 2000) and human body growth (Barile and Rastall, 2013) of the infants, anti-inflammatory (Kunz and Rudloff, 2008), antioxidant (Tulika and Desh, 2014), bifidus factors (Kitaoka, 2012), increased lactation in women etc. In ancient times there was a limited variety of cow breeds with minimal milk production. Even then the benefits of the cow milk cannot be overruled. The ancient medical literature of Ayurveda and Charak Samghita clearly defines the medicinal properties of cow milk but there was no indication in the literature as to which part of the milk that is protein, fat or carbohydrates is actually responsible for its medicinal values. Although it was thought that a combination of the above contents in a specific ratio of all the contents is jointly responsible for the biological activity. Later with the advent of science it was found the the cow milk contains 87% water, 3.5% protein, 4% fats and 4.8% carbohydrates, besides the other important micronutrients like vitamins, minerals, .8%. It also contains lactose and oligosaccharides which were not known in the vedic period. with the recent encouragement given by the government of India for the enhancement of national dairy products development (NPDD) and rashtriya gokul mission (RGM) which resulted in the enhancement of production of cow milk and boom in the number of new species of cow . Hence with the evolution in the number of various cow species it becomes necessary to investigate the milk content of various cow species which depends on the geographical habitat and the fodder intake of the cow.

The main breeds which produce large volumes of milk in India are jersey (Punjab), frazier (Punjab), sahiwal (Haryana), tharparkar (Rajasthan), kankraz (Rajasthan), rathi (Rajasthan), shyamadhenu (U.P) and A2 cow etc. it was found after rigorous research that the ratio of milk content (Protein, fat and oligosaccharide) vary with the variety of the species . It was concluded by the results obtained during these researches that the oligosaccharides were the main constituents responsible for the bio-efficacy of the milk. It was also found that the oligosaccharide content of milk of different species were structurally different. The oligosaccharides are sugar chains comprising of two to fourteen mono saccharides linked together by o-glycosidic linkages in a straight of branched chains the main constituent of a milk oligosaccharide are Glc, Gal, GlcNAc, GalNAc, fucose and sialic acid which are linked together at different positions of a monosaccharide i.e. position 1-,2-,3- or 4-hydroxy group of a particular monosaccharide. The other variations which causes the differentiation in the oligosaccharide structure is α and β configuration of the glycosidic linkage there were various methods which were adopted by different scientist which mainly are Urashima et. al (1997), Smith et. al (1978), Egge et. al (1983), Weiruszeski et. al (1985), Kobata et. al (1970), Modified method of Kobata and Ginsberg by D. Deepak et. al. (2018). Basically these methods were used for isolation of milk oligosaccharides from human milk, later when more studies performed on the oligosaccharide content of rare and common species of cow by D. Deepak et.al following method were adopted.

A2 Cow

Shyamadhenu Black Cow

Jarsi Cow

Frazier Cow

Sahiwal Cow

Tharparkar Cow

Kankrej Cow

Rathi Cow

Process for Isolation of Milk Oligosaccharides

Isolation of Milk Oligosaccharides by D. Deepak et. al. (2018)

Cow milk (10 litres)

↓ Equal amount of ethanol (10 litres) was added & filtered by glass wool followed by centrifugation (5000 rpm) at -4°C and filtered by glass wool

Lipid Layer (Discarded)

Supernatant

↓ Was precipitated by addition of 68% ethanol and separated by centrifugation

Supernatant

It was filtered through a micro filter (0.2µ) to remove remaining lactose. It was then lyophilized

Protein and Lactose Residue (**Discarded**)

↓

Lyophilized Material

(Mixture of oligosaccharides)

↓ Was then fractionated on a Sephadex G-25 column, eluted with triple distilled water at flow rate 3ml/min. Repeated Sephadex Chromatography resulted into x gm of MOs.

Carbohydrate Containing Fractions

(Fractions were pooled, lyophilized and analyzed by HPLC)

↓

Analytical HPLC

The carbohydrate fractions were eluted with TDW (containing 0.1%TFA & CH_3CN) at a flow rate 1ml/min., to check homogeneity of the oligosaccharide mixture. The elution was monitored by UV absorbance at 220 nm (Table 3).

Chemical Transformation

Oligosaccharide mixture was acetylated with Ac_2O and pyridine converting free sugar into non-polar acetyl derivatives which were resolved nicely on TLC and were separated by column chromatography over silica gel which resulted in the isolation of four chromatographically pure compounds.

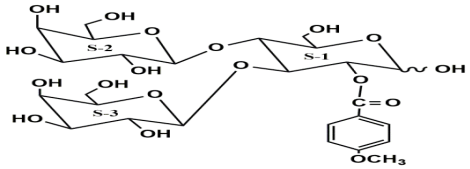
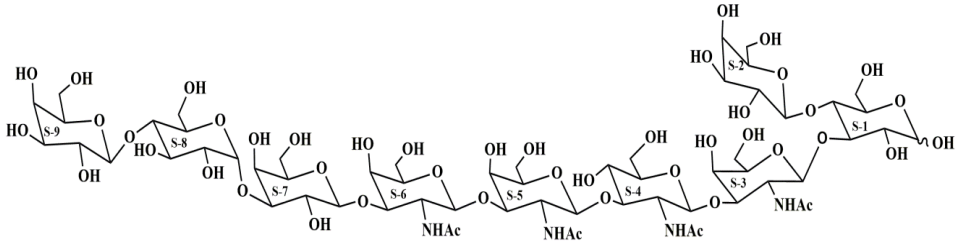
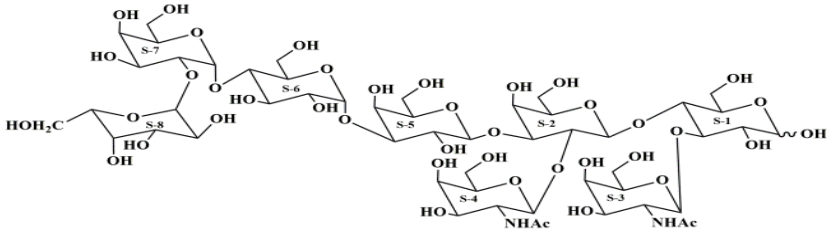
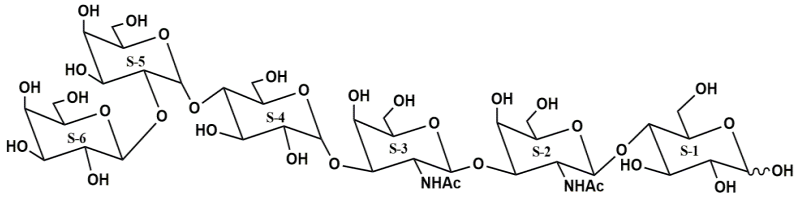
↓

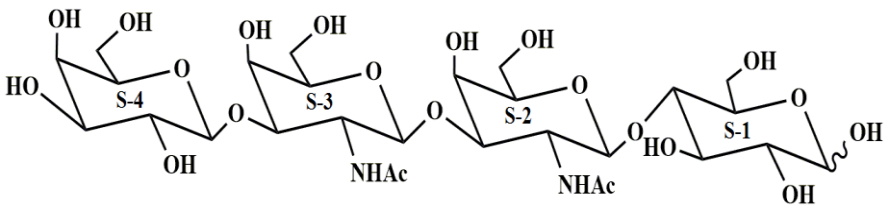
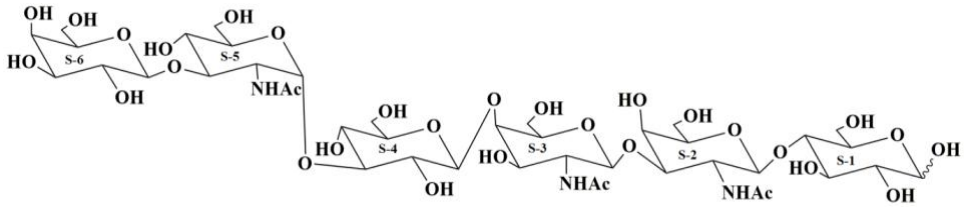
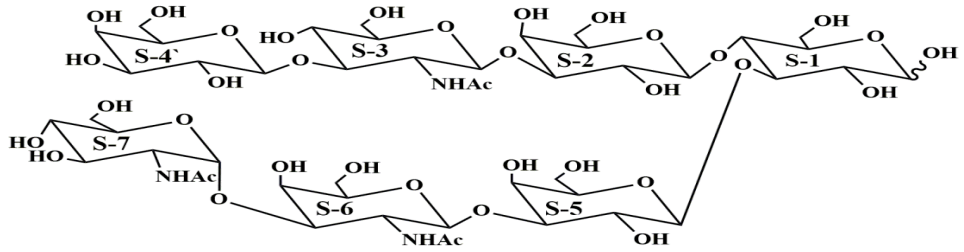
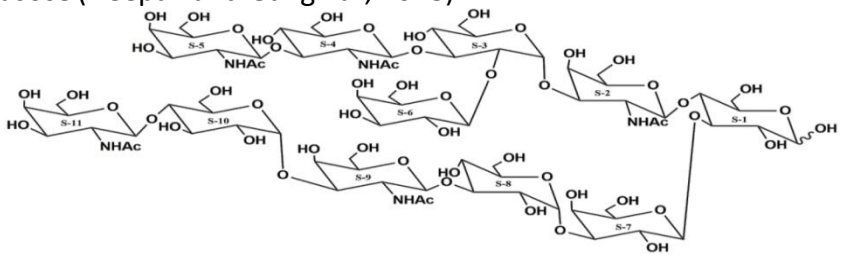
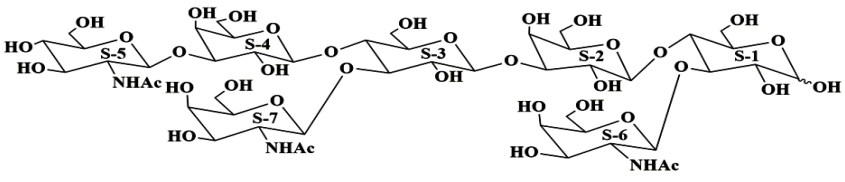
Deacetylation

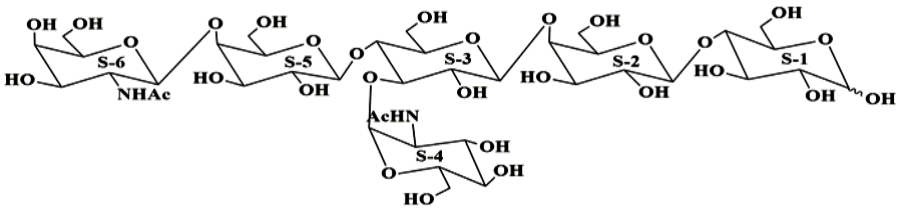
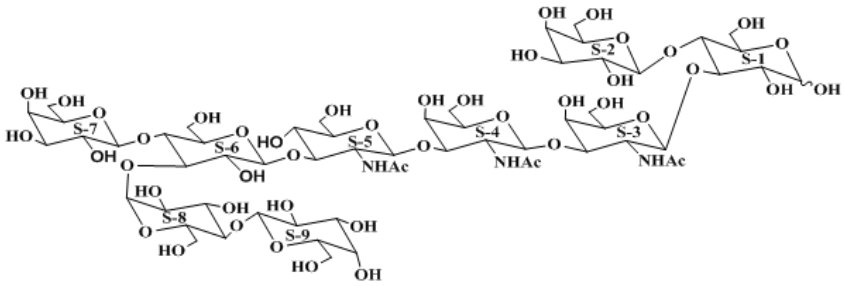
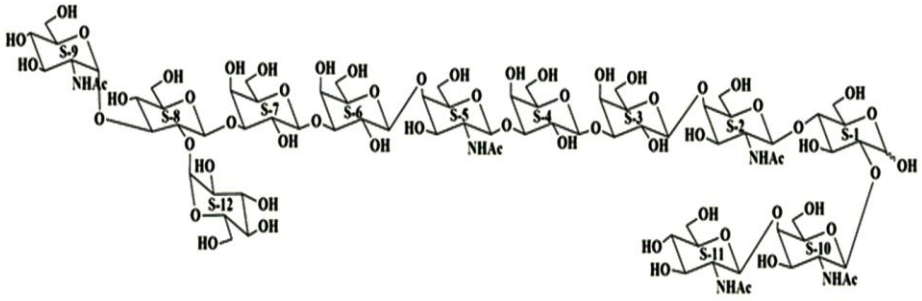
The chromatographically pure acetylated Milk oligosaccharides were deacetylated by dissolving them in acetone & NH_4OH and left overnight. Ammonia was removed under reduced pressure and the compound was washed with $CHCl_3$ and was finally freeze dried giving the deacetylated natural milk oligosaccharides.

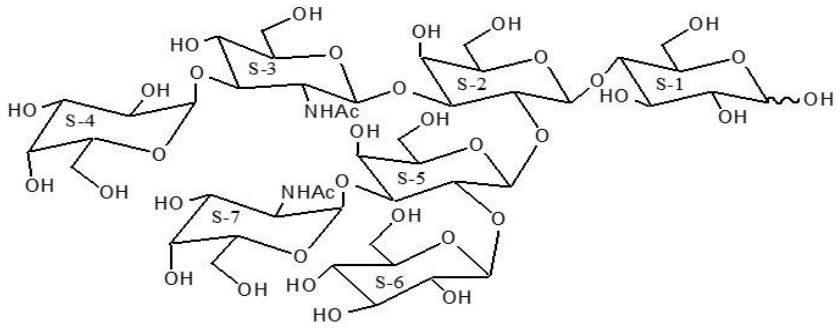
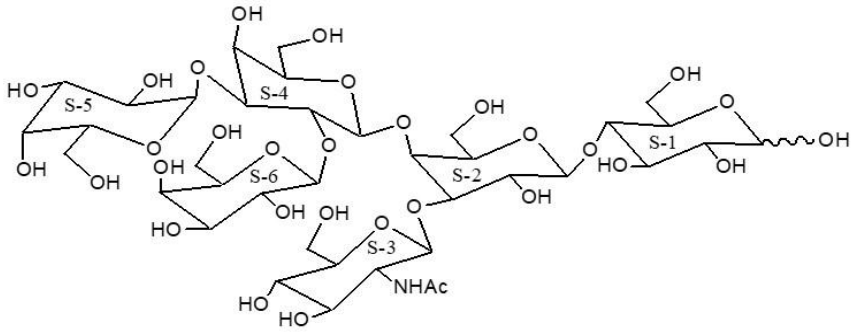
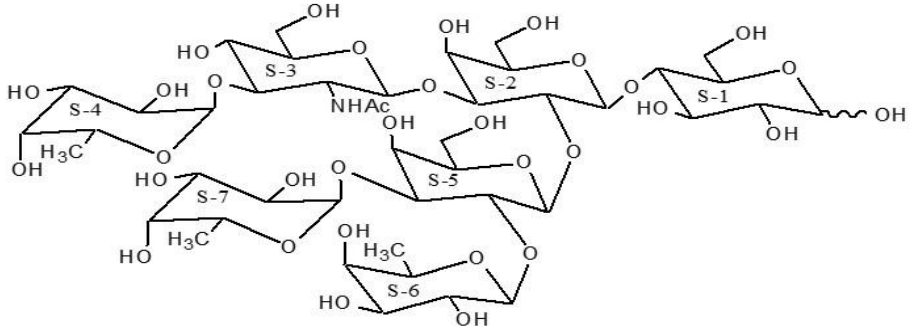
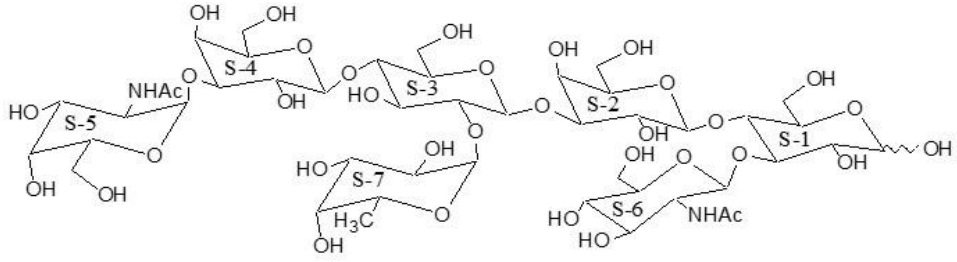
After obtaining the crude oligosaccharide mixture which still contains glycoproteins , proteins and lactose as artifacts, they were removed by microfiltration and gel filtration , chromatography (Sephadex chromatography) which resulted into the isolation of pure oligosaccharide mixture which is further purified by a combination of various chromatographic techniques for obtaining the chromatographically pure oligosaccharide . The structure of these purified oligosaccharides were elucidated using numerous physicochemical techniques such as NMR (^{1}H,^{13}C) 2-D NMR (COSY,TOCSY,HSQC,HMBC) experiments along with mass spectrometry. The structures of these oligosaccharides are given as under:

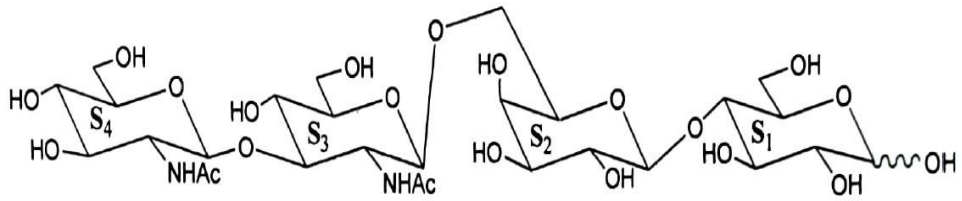
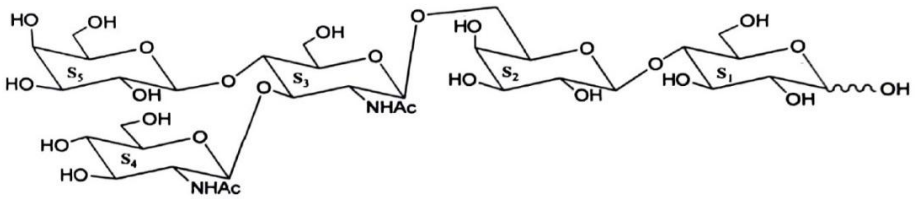
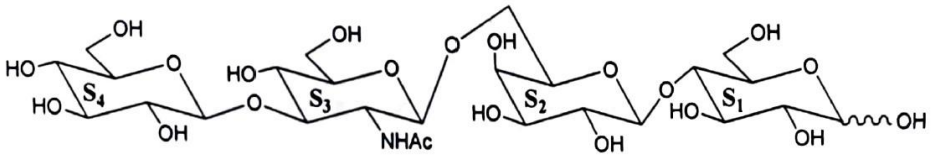
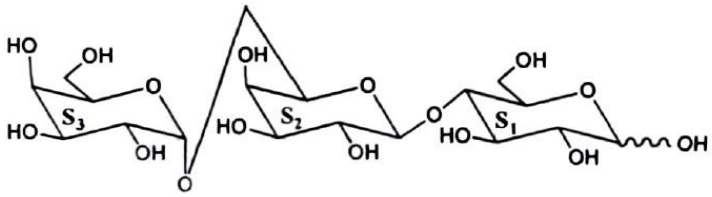
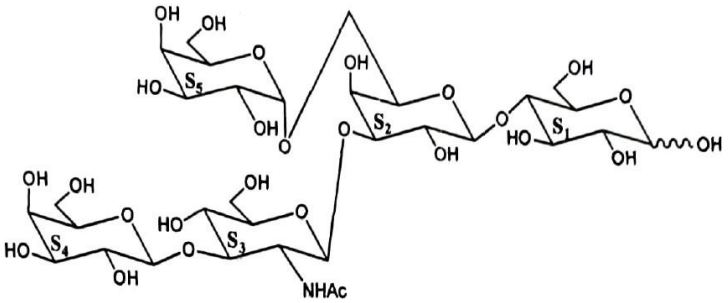
Milk oligosaccharides isolated from various cow species

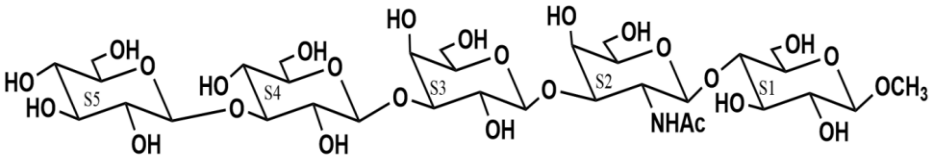
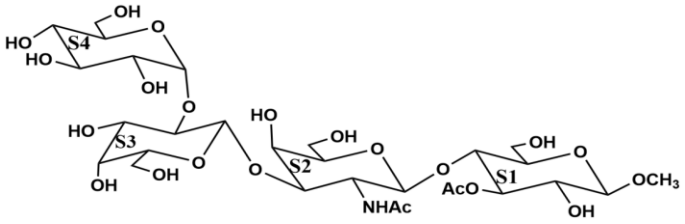
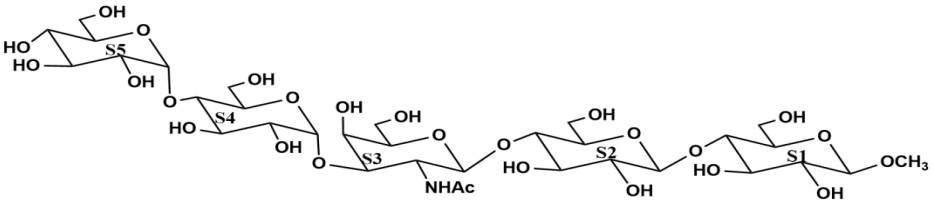
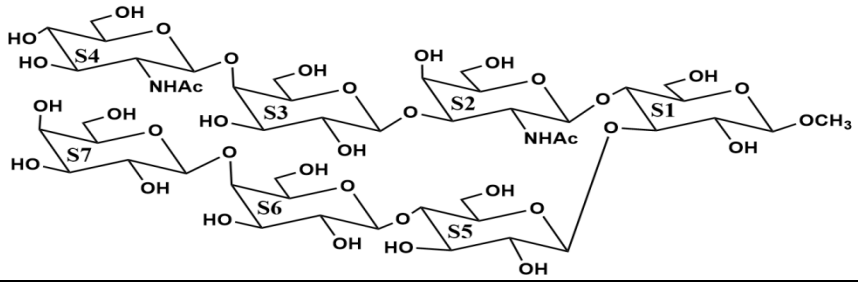
Name of Milk Oligosaccharide and its Structure	Animal
1. Aurose (Gunjan and Deepak, 2019) 	Cow Colostrum (Jarsi)
2. Tarose (Desh Deepak, 2017) 	Cow Colostrum (Jarsi)
3. Ososе (Desh Deepak, 2017) 	Cow Colostrum (Jarsi)
4. Tososе (Desh Deepak, 2017) 	Cow Colostrum (Jarsi)

5. Asose (Deepak et al., 2020) 	Black Cow Milk
6. Urose (Deepak and Gangwar, 2018) 	Black Cow Milk
7. Ausose (Deepak and Gangwar, 2018) 	Black Cow Milk
8. Tausose (Deepak and Gangwar, 2018) 	Black Cow Milk
9. Rusose (Khan et al., 2018) 	Lal Muha Cow milk

10. Usose (Khan et al., 2018) 	Lal Muha Cow milk
11. Taurose (Khan et al. 2019) 	Lal Muha Cow milk
12. Uruose (Khan et al. 2019) 	Lal Muha Cow milk

13. Bososе (Tripathi et al., 2011) 	Chauri cow milk
14. Unninose (Tripathi 2012) 	Chauri cow milk
15. Nakose (Trpathi and Deepak 2012) 	Chauri cow milk
16. Nienose (Tripathi and Deepak, 2012) 	Chauri cow milk

17. Indicose (Gunjan et al., 2016) 	Black cow milk
18. Indose (Khan et al., 2017) 	Black cow milk
19. Indinose (Khan et al., 2017) 	Black cow milk
20. Bosnose (Gunjan et al., 2017) 	Black cow milk
21. Dicusose (Gangwar et al., 2017) 	Black cow milk

22. Tharoside (Deepak and Sharma, 2022) 	Tharparkar Cow milk
23. Parkoside (Deepak and Sharma, 2022) 	Tharparkar Cow milk
24. Karoside (Deepak and Sharma, 2022) 	Tharparkar Cow milk
25. Arkaroside (Deepak and Sharma, 2022) 	Tharparkar Cow milk

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