Journal of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)

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Homology modeling of novel Hypothetical protein VCA0739 (YZ39_VIBCH) from pathogenic microorganism *Vibrio cholerae.*

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Article info

Article history:

Received 1 July 2016
Revised 15 July , 2016
Accepted 10 August 2016
Available online 31 August, 2016

Keywords:

Homology Modeling,
Hypothetical Protein,
Cholera
Protein data bank

ABSTRACT

We determined the 3D model structure of the hypothetical protein (YZ39_VIBCH protein V) protein from Vibrio cholerae. ESyPred3D software more accurately developed the protein model than swiss model for our query protein V. In addition, the plant medicinal compound Chicoric acid found more affinity for hypothetical protein (YZ39_VIBCH protein V) as compare to Doxycycline antibiotic. Therefore Chicoric acid can be act as alternative medicine for treating the cholera disease.

INTRODUCTION

Homology modeling is a tool to predict 3D structure of those proteins whose structures are not define by X-ray or NMR method (1). The 3D structures of proteins are stored in to RCSB protein data bank. There is large number of protein sequences are available in sequence databases however only structure of few proteins are determined. Therefore, Homology modeling plays a major role in prediction of the 3D structure of Proteins. Structural genomics projects have determined 3D structures of the proteins via high-throughput methods like structure modeling, threading as well as combination of experimental and modeling approaches (2,3). Currently, the number of protein sequence entries has increased to 65,378,749 in sequence databases such as UniProtKB/TrEMBL, however, 3D structures of only 121654 proteins are deposited in RCSB protein data bank. Therefore, the structural genomics projects are developed to resolve the 3D structures of several proteins from the whole genome rather than working with one protein at a time. Their major goals are to determine large number of protein structures with novel protein folds. Although, the structural genomics projects provide new protein structures in RCSB protein databank, they open new challenges of assigning function and functional sites for these proteins (2,3). Majority of these proteins fail to produce sequence and structure homology with the proteins of known function. In addition, a number of SG proteins do not show a fold level similarity with known proteins. Over one third of the structures from the structural genomics initiatives have no additional experimental data to infer functions and annotated as "hypothetical proteins". Therefore, prediction of function and functional sites in SG proteins is an important and non-trivial task. Many of these hypothetical proteins are present in pathogenic microorganisms and can act a potential drug

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targets (4,5). Computational tools have been extensively used to characterize proteins (by homology modeling)(1, 3, 20, 21), drug discovery or ligand prediction (by Molecular Docking) (2,4-10) and structure characterization of the ligands (by Molecular modeling)(11,12). Therefore, inspired by these techniques, we explored the use of homology modeling and molecular docking in order to predict the structure of unknown protein in ***Vibrio cholera*** and to design new drugs against this microorganism.

Vibrio cholera (also *Kommabacillus*) is a gram negative comma-shaped bacterium with a polar flagellum that causes cholera in humans (13). *V. cholerae* and other species of the genus *Vibrio* belong to the gamma subdivision of the Proteobacteria (13). *V. cholerae* was first isolated as the cause of cholera by Italian anatomist Filippo Pacini in 1854. But his discovery was not widely known until Robert Koch, working independently thirty years later, publicized the knowledge and the means of fighting the disease. **Cholera** (frequently called **Asiatic cholera** or **epidemic cholera**) is a severe diarrheal disease caused by the bacterium ***Vibrio cholerae*** (14,15)**.**

Transmission to humans is by water or food (16). It was long assumed that its natural source is humans, but some evidence suggests that it is the aquatic environment (16). Its toxic compounds acts on the Mucosal Epithelium and having more fatalness. In its extreme manifestation, cholera is one of the most rapidly fatal illnesses known. A healthy person may become hypotensive within an hour of the onset of symptoms and may die within 2-3 hours if no treatment is provided. C**linical description:** cholera begins with sudden onset of massive diarrhea (17,18). The patient may lose gallons of protein-free fluid and associated electrolytes, bicarbonates and ions within a day or two (18). Therefore, there is need to develop new and effective drugs (19). Due to changes in the characteristics of cholera bacteria there is further need for finding of novel drug target (19). The major goal is the prevention of disease in early stage of *V . cholera* life cycle. Therefore implementation of new methodology for fast drug target finding. Its genome has been sequenced (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=gvc) and about 628 of its proteins are known to be conserved hypothetical proteins. The current case study determines structure of one of the hypothetical protein from Cholera bacteria and further drug design.

MATERIALS AND METHODS

Input sequence

The protein sequence of hypothetical protein VCA0739 from *Vibrio Cholera* was obtained from gene bank (Accession number: *NP_233126.1*) and subjected to BLAST for sequence alignment. Furthermore, the sequence was furnished as query sequence for homology modeling.

>gi|15601495|ref|NP_233126.1| hypothetical protein VCA0739 [Vibrio cholerae O1 biovar El Tor str. N16961]
MENLAELPSPWFVYLVRCANNALYCGITTDVSRRFA QHQKGRGAKALRGKGPLELVWSLPVADGKSAALK LEYRIKALSKSQKEALVAGMARIDQLEIIFQ

Sequence Comparison

The sequence of VCA0739 from *Vibrio Cholera* subjected to BLAST tool for finding putative match for the query sequence. Sequence matching will help in classification of the query protein. The search was conducted against non-redundant and pdb databases. Further, the top matching sequence

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and pdb were selected as template for homology modeling step.

Homology modeling

The protein sequence was subjected for comparative homology modeling via Swiss model (Arnold et al, 2006; Kiefer et al, 2009; Peitsch et al, 1995; Lorenza et al, 2008) and ESyPred3D (via Modeller 6v2) software's (Lambert et al, 2002) to generate putative 3D model. The Swiss model performs the sequence alignments and searches the putative template protein for generating the 3D model for query sequence. The ESyPred3D has been incorporated with Modeller (version 6v2) program for generating the putative 3D model. All the modeling parameters were set to be default. The model structure was further verified by PROCHECK and PROSA analysis.

Energy minimization by GROMOS96

The model structure was further optimized by energy minimization via GROMOS96, implemented in Swiss pdb viewer software. GROMOS96 performs the molecular dynamics of all the bonded and non bonded atoms within the model structure and obtain the minimum potential energy.

Functional site prediction

We subjected the model structure to different function and functional site prediction servers *e.g.* DALI, BLAST, PSI-BLAST, PROFUNC, Q-SITE FINDER and PROSITE. The BLAST and PSI-BLAST were used for function verification. On the other hand, the PROFUNC and Q-SITE FINDER were used for structure based functional site prediction.

Inhibitor prediction

Finally, the inhibitor compounds were detected for modeled hypothetical protein VCA0739 in order to decrease its activity. We obtained natural plant anti viral inhibitors compounds from literature. Their SMILES strings were obtained from Pubchem database and 3D structures were generated by CORINA server. The compounds were docked against the modeled structure of hypothetical protein VCA0739.

RESULTS

We predicted the putative 3D model structure of its one of the hypothetical protein (YZ39_VIBCH protein V) from ***Vibrio cholerae.*** We implemented the homology modeling tools for 3D structure prediction. The different state of the art method, like BLAST, PSI BLAST (10 iteration), Phylogeny tree and UniPort, analysis provided clue about the putative endonuclease function of hypothetical protein (YZ39_VIBCH protein V).

With non-redundant protein sequence (nr)

1: putative endonuclease containing a URI domain [Vibrio cholerae TMA 21]

Sequence ID: ref|ZP_04403649.1|Length: 101Number of Matches: 1

Related Information
Range 1: 1 to 101GenPeptGraphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Method	Identities	Positives	Gaps
202 bits(515)	2e-65	Compositional matrix adjust.	98/101(97%)	99/101(98%)	0/101(0%)

```
Query  1    MENIAELPSFWFVYLVRCANNALYCGITTDVSRRFAQHQKGRGAKALRGKGPLELVWSLP  60
            ME IAELPSFWFVYLVRCANNALYCGITTDVSRRFAQHQKG GAKALRGKGPLELVWSLP
Sbjct  1    MEIIAELPSFWFVYLVRCANNALYCGITTDVSRRFAQHQKGSGAKALRGKGPLELVWSLP  60

Query  61   VADGKSAALKLEYRIKALSKSQKEALVAGMARIDQLEIIFQ  101
            VADGKSAALKLEYRIKALSKSQKEALVAGMARI+QLEIIFQ
Sbjct  61   VADGKSAALKLEYRIKALSKSQKEALVAGMARIEQLEIIFQ  101
```

With pdb databank database (pdb)

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1: Chain A, Solution Nmr Structure Of The Upf0213 Protein Bh0048 From Bacillus Halodurans.

Northeast Structural Genomics Target Bhr2.

Sequence ID: pdb|1ZG2|A Length: 107 Number of Matches: 1

Structure-3D structure displays

Range 1: 1 to 84 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
76.6 bits(187)	1e-18	Compositional matrix adjust.	37/87(43%)	53/87(60%)	5/87(5%)

```
Query  4   LAELPSPWFVYLVRCANNALYCGITTDVSRRFAQHQKGRGAKALRGKGPLELV--WSLPV  61
           +A  P   +VY++ C + + Y G TTDV RR  +H  G+GAK  RG+GP  LV  W+ P
Sbjct  1   MAGDPMNHYVYILECKDGSWYTGYTTDVDRRIKKHASGKGAKYTRGRGPFRLVATWAFP-  59

Query  62  ADGKSAALKLEYRIKALSKSQKKALVA  88
           K  A++ EY +K LS+ +KK LV+
Sbjct  60  --SKEEAMRWEYEVKHLSRRKKEQLVS  84
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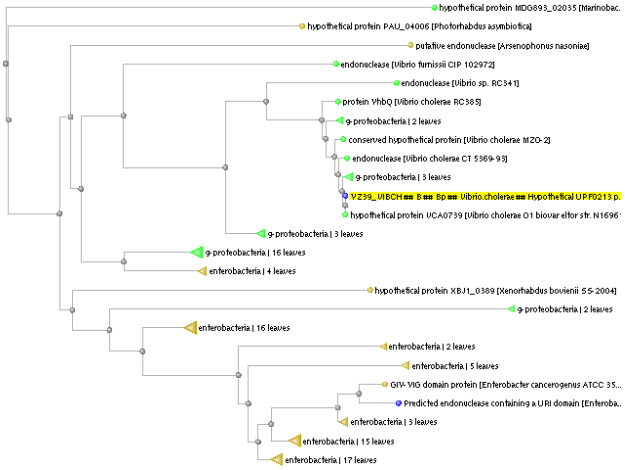

PSI-BLAST RESULTS

Run PSI-Blast iteration 11 with max

Sequences producing significant alignments with pattern at position and E-value BETTER than threshold

Sequences producing significant alignments with pattern at position and E-value BETTER than threshold

Accession	Description	Max score	Total score	Query coverage	E value	Lin
ZP_01679145.1	conserved hypothetical protein [Vibrio cholerae V52] >ref|YP_001215514.1| hypothetical protein VC0395_0675 [Vibrio cholerae O395] >ref|ZP_04411718.1| putative endonuclease containing a URI domain [Vibrio cholerae TM 11079-80] >ref|ZP_06036276.1| endonuclease [Vibrio cholerae RC27] >sp|A5EZS6.1|Y675_VIBC3 RecName: Full=UPF0213 protein VC0395_0675/VC395_A0575 >gb|EAX63978.1| conserved hypothetical protein [Vibrio cholerae V52] >gb|ABQ18868.1| conserved hypothetical protein [Vibrio cholerae O395] >gb|ACP11409.1| conserved hypothetical protein [Vibrio cholerae O395] >gb|EEO05293.1| putative endonuclease containing a URI domain [Vibrio cholerae TM 11079-80] >gb|EEY41814.1| endonuclease [Vibrio cholerae RC27]	106	106	100%	1e-21	
ZP_06048456.1	endonuclease [Vibrio cholerae CT 5369-93] >gb|EEY52395.1| endonuclease [Vibrio cholerae CT 5369-93]	105	105	100%	1e-21	
ZP_01976995.1	conserved hypothetical protein [Vibrio cholerae MZO-2] >gb|EDM55911.1| conserved hypothetical protein [Vibrio cholerae MZO-2]	105	105	100%	1e-21	
NP_233126.1	hypothetical protein VCA0739 [Vibrio cholerae O1 biovar eltor str. N16961] >ref|ZP_01675374.1| protein YhbQ [Vibrio cholerae 2740-80] >ref|ZP_01950031.1| conserved hypothetical protein [Vibrio cholerae 1587] >ref|ZP_01972084.1| conserved hypothetical protein [Vibrio cholerae NCTC 8457] >ref|ZP_01973408.1| conserved hypothetical protein [Vibrio cholerae B33] >ref|ZP_01982522.1| conserved hypothetical protein [Vibrio cholerae 623-39] >ref|YP_002812316.1| hypothetical protein VCM66_A0697 [Vibrio cholerae M66-2] >ref|ZP_04395613.1| putative endonuclease containing a URI domain [Vibrio cholerae BX 330286] >ref|ZP_04399519.1| putative endonuclease containing a URI domain [Vibrio cholerae B33] >ref|ZP_04405847.1| putative endonuclease containing a URI domain [Vibrio cholerae RC9] >ref|YP_002876339.1| putative endonuclease containing a URI domain [Vibrio cholerae MJ-1236] >ref|ZP_04919769.1| conserved hypothetical protein [Vibrio cholerae V51] >ref|ZP_05239249.1| conserved hypothetical protein [Vibrio cholerae MO10] >ref|ZP_05420447.1| putative endonuclease containing a URI domain [Vibrio cholera CIRS 101] >ref|ZP_06028526.1| endonuclease [Vibrio cholerae INDRE 91/1] >ref|ZP_07009341.1| conserved hypothetical protein [Vibrio cholerae MAK 757] >sp|Q9KLK4.1|Y3539_VIBCH RecName: Full=UPF0213 protein VC_A0739 >gb|AAF96638.1| conserved hypothetical protein [Vibrio cholerae O1 biovar El Tor str. N16961] >gb|EAX60209.1| protein YhbQ [Vibrio cholerae 2740-80] >gb|EAY33494.1| conserved hypothetical protein [Vibrio cholerae 1587] >gb|EAZ49615.1| conserved hypothetical protein [Vibrio cholerae V51] >gb|EAZ72619.1| conserved hypothetical protein [Vibrio cholerae NCTC 8457] >gb|EAZ79007.1| conserved hypothetical protein [Vibrio cholerae B33] >gb|EDL72791.1| conserved hypothetical protein [Vibrio cholerae 623-39] >gb|ACP07659.1| conserved hypothetical protein [Vibrio cholerae M66-2] >gb|EEO11252.1| putative endonuclease containing a URI domain [Vibrio cholerae RC9] >gb|EEO17424.1| putative endonuclease containing a URI domain [Vibrio cholerae B33]	105	105	100%	1e-21	G

Tree view

Molecular Modeling

The query sequence was subjected to Swiss model and Modeller 6v2 (EsyPred3D) softwares for 3D structure prediction. The structure was predicted based on the template pdb 1ZG2 (chain A) (with sequence Identity 35.40% (Modeller6v2), 36.66 % (Swiss model, fig.2)). The Ramachandran plot verification through Procheck revealed that Modeller 6v2 (EsyPred3D) more precisely predicted the 3D model structure for hypothetical protein (YZ39_VIBCH protein V) with 95.90 % of residues are in favored (A, B, L) and additional allowed (a, b, l, p) regions (Fig.1). On the other hand Swiss model predicted only 93.50 % of residues (Fig.2). Plot shows more than 90% residues have position in most favored region and having very less bad non-bonded interactions(Fig.3).

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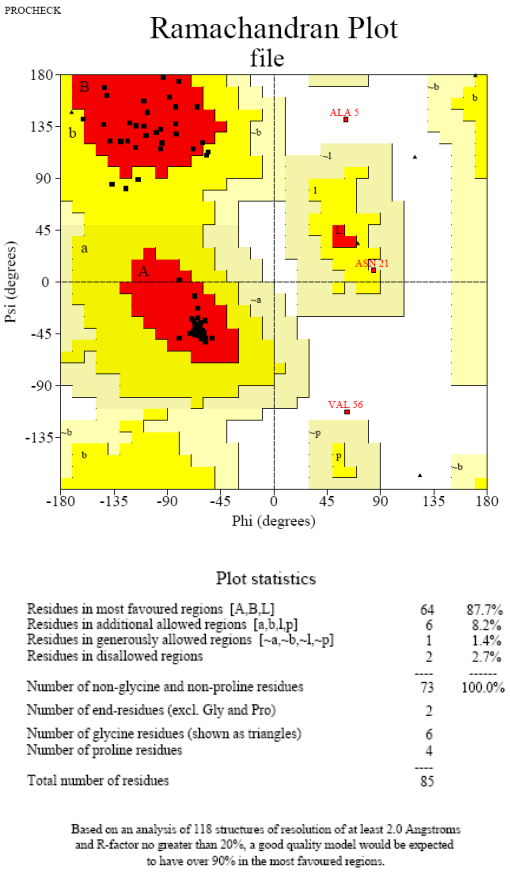
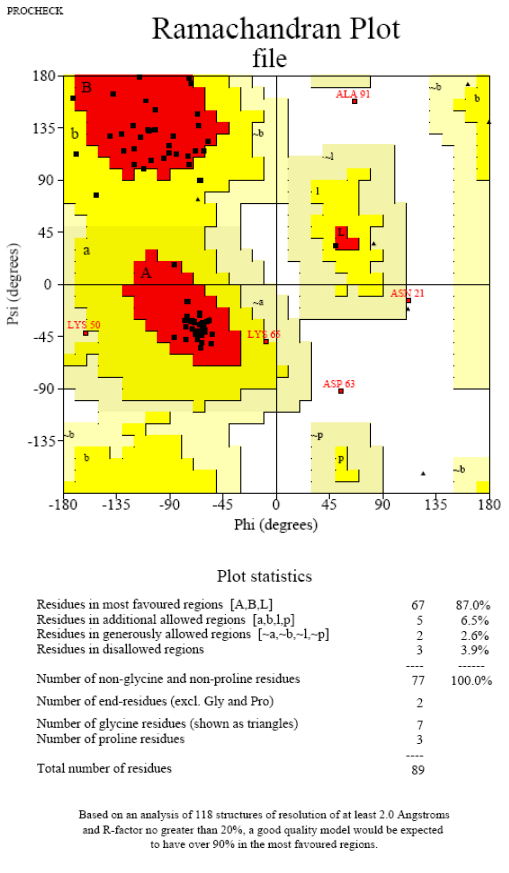


Plot statistics

Residues in most favoured regions [A,B,L]	64	87.7%
Residues in additional allowed regions [a,b,l,p]	6	8.2%
Residues in generously allowed regions [~a,~b,~l,~p]	1	1.4%
Residues in disallowed regions	2	2.7%
Number of non-glycine and non-proline residues	73	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	6	
Number of proline residues	4	
Total number of residues	85	

Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

Fig.1 : Ramachandran plot analysis for EsyPred3D model.

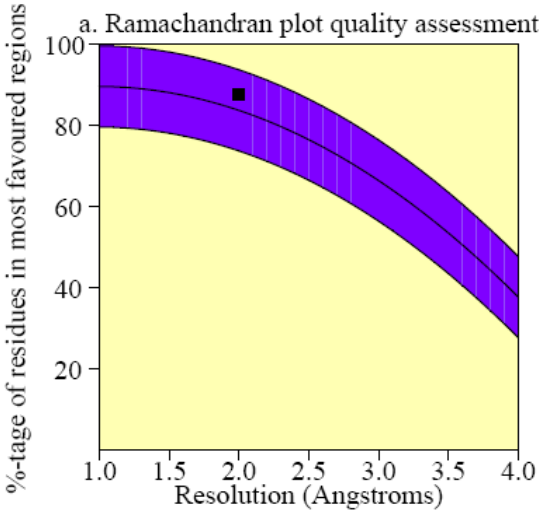


Plot statistics

Residues in most favoured regions [A,B,L]	67	87.0%
Residues in additional allowed regions [a,b,l,p]	5	6.5%
Residues in generously allowed regions [~a,~b,~l,~p]	2	2.6%
Residues in disallowed regions	3	3.9%
Number of non-glycine and non-proline residues	77	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	7	
Number of proline residues	3	
Total number of residues	89	

Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

Fig. 2: Ramachandran plot analysis for Swiss model.



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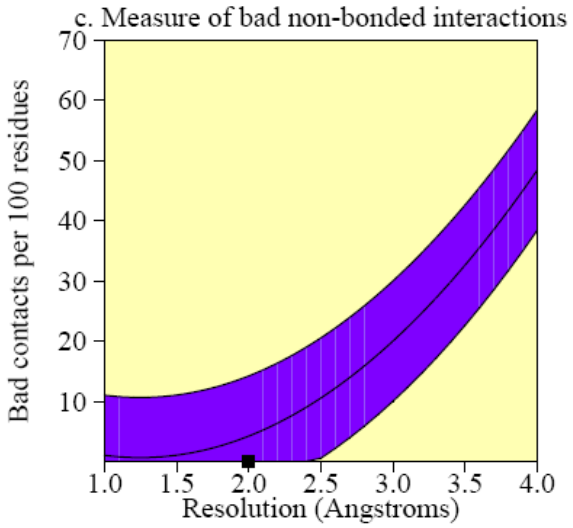
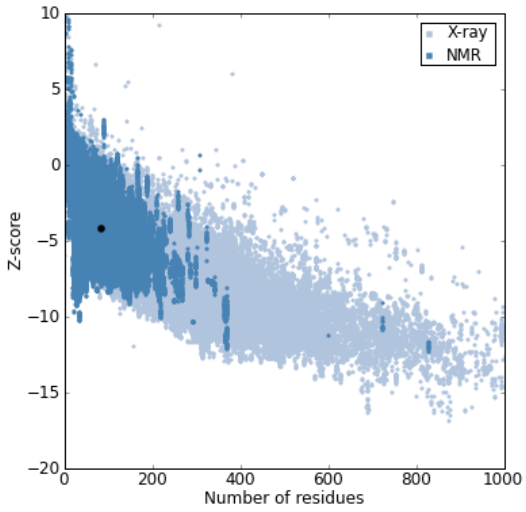


Fig.3: Plot quality for YZ39_VIBCH model from EsyPred3D model.

ProSA analysis also concluded that model through Modeller 6v2 (EsyPred3D) resides in NMR region of the plot with Z score of -4.21(Fig.4) but Swiss model not able to detect any similarity with any region (NMR or X-ray). It also determined the energy statues of the residues and found lower energy for almost all residues(Fig.4).



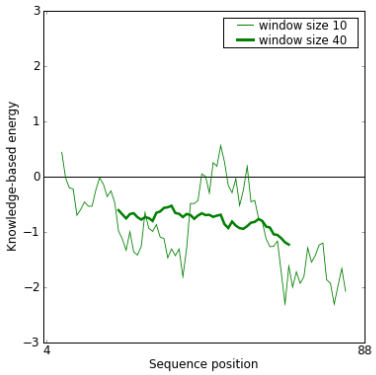


Fig.4: PROSA analysis.

The model by Modeller 6v2 was further stabilized by the energy from 371.703 kJ/mol to -2488.739 KJ/mol via GROMOS96 force field. In addition, Secondary structure prediction of hypothetical protein (YZ39_VIBCH protein V) revealed that the modeled structure consist of Helix-Strand-Coil like structure (Fig.5).

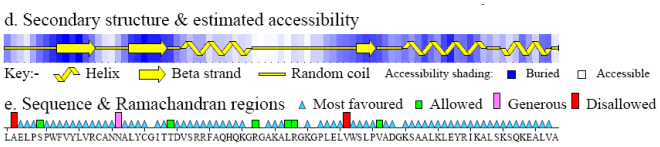


Fig.5: Secondary structure prediction for model hypothetical protein

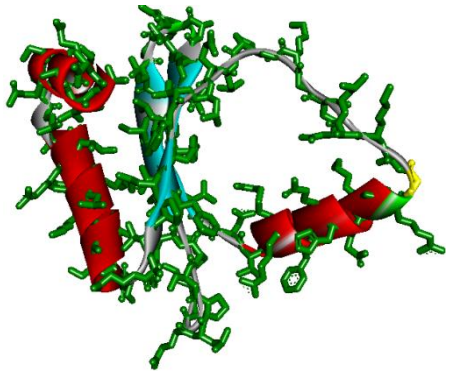


Fig.6 : Model structure of hypothetical protein (YZ39_VIBCH protein V).

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Functional site residues

The model from Modeller 6v2 was further subjected to functional genomics study. The DALI and PINTS failed to detect any significant match with model structure of hypothetical protein (YZ39_VIBCH protein V) but Q-site finder server enumerated the putative active site residues R33 A36 Q37 K40 G41 A44 K45 A46 L47 R48 with site volume of 195 cubic Å. The grid box coordinates of active site are Min Coordinates: (-1, -19, -11), Max Coordinates: (12, -5, 2). Profunc server also predicted putative active site residues Y14 G26 E72 R74 V187 with RMSD of 1.01 Å and confirmed the Endonuclease function of hypothetical protein (YZ39_VIBCH protein V). Based on our study we predicted that the hypothetical protein (YZ39_VIBCH protein V) may helps in DNA repair mechanism of ***Vibrio cholerae*** during DNA replication. Therefore it can act as the potential Drug target to prevent the infection of ***Vibrio cholerae.*** The structure and functional site prediction of hypothetical protein (YZ39_VIBCH protein V) may be useful for biologist to design drug against cholera disease.

Docking with Lead compounds

Docking via Patchdock produced higher docking score for plant medicinal compound Chicoric acid (score 5270, Table 1) as compare to the well know first line anti-cholera antibiotic Doxycycline (score 4490, Table 1)(Fig.7). Both the inhibitors bind at the cavity of modeled hypothetical protein (YZ39_VIBCH protein V), however Chicoric acid show higher affinity as compare to Doxycycline antibiotic. Both the compounds form hydrogen bonds with V187 and I186(Fig.7). However, Chicoric acid form additional hydrogen dong with R74 which may provide higher affinity for hypothetical protein (YZ39_VIBCH protein V) protein.

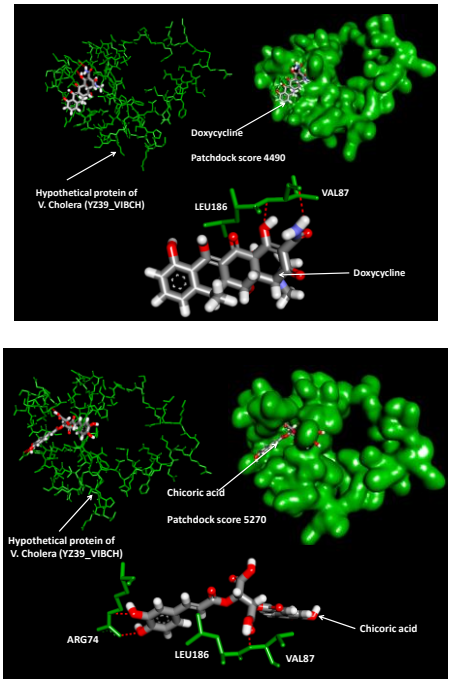

Fig.7: Patchdock docking of Chicoric acid and Doxycycline with hypothetical protein (YZ39_VIBCH protein V) protein.

Table 1: Patchdock score for Plant medicinal compounds and Antibiotics against modeled structure of protein V

Rank	Plant Medicinal compounds	Patchdock score
1	Chicoric_acid	5270
2	Abyssinone	4862
3	Decadienal	4388
4	Capsaicin	4344
5	Tannic acid	4336
6	Rosmarinicacid	4336
7	Warfarin	4012
8	Cyanidin.	3868
9	Epicatechin	3758

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10	Ellagicacid	3240
11	Indolecarbinol	2932
12	Hydroxycinnamicacid	2876
13	Ferulic_acid.	2682
14	Gallicacid	2600
15	Pyrogallol	2484

Rank	Antibiotics (First line for V. Cholera)	Score
1	Doxycycline	4490
2	Tetracycline	4432
3	chloramphenicol	3400

It establishes the hypothesis that Chicoric acid may act as potential drug against *V. Cholerae*. The Chicoric acid is found in *Echinacea pupurea* plant. It is a caffeic acid derivative, belonging to the group of polypfenols. Chicoric acid helps in improving immune system and promotes phagocytosis. This is the process whereby white blood cells and lymphocytes attack and destroy pathogens. Chicoric acid stimulates T-cell activation, stimulates healing of wounds and reduces the inflammation in arthritis. Chicoric acid increases the production of interferon, immunoglobulin and other chemicals important for the immune system. Studies have indicated that chicoric acid can inhibit the penetration of viruses in cells. Chicoric acid also acts as an antioxidant by preventing the oxidation of collagen and cells.

DISCUSSION

In our study, we determine the 3D model structure of the hypothetical protein (YZ39_VIBCH protein V) protein from *Vibrio cholerae*. We investigate that ESyPred3D software develop more accurate protein model for our query sequence as compare to swiss model. It also verifies by PROCHECK and ProSA analysis. These methods confirm the accuracy of our protein model. Structure based functional site prediction methods enumerate the putative amino acid residues from our protein model of hypothetical protein (YZ39_VIBCH protein V) protein. In extension of our study, we determine the potential drug candidate for our model protein via docking. In addition, the plant medicinal compound Chicoric acid shows more affinity for hypothetical protein (YZ39_VIBCH protein V) protein as compare to Doxycycline. As Chicoric acid is plant origin therefore it may have no side effect and easily available. There this compound can be act as substitute for treating the cholera disease. Therefore, the computational method has power to reduce the time and cost for drug development.

ACKNOWLEDGEMENTS

Indu Chaturvedi greatly acknowledges Indian Science and Technology Foundation (ISTF) for providing all financial supports and computer facility for running docking calculations.

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Journal of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)

JOS

Homology Modeling of Hypothetical protein (UPF0213 protein Y) from pathogenic microorganism *Yersinia pestis*

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Article info

Article history:

Received 11 July 2016
Revised 20 July , 2016
Accepted 15 August 2016
Available online 31 August, 2016

Keywords:

Plague,
Homology Modeling,
Hypothetical Protein

ABSTRACT

Yersinia pestis is a Gram-negative rod-shaped bacterium that can infect humans and other animals and cause deadly disease "Plague". We determined the putative 3D structure of hypothetical protein (UPF0213 protein Y) from Yersinia pestis via Comparative homology modeling tools and predicted to have function like Excinuclease enzyme which may help in DNA repair mechanism of Yersinia pestis. This may act as the potential target to prevent the Plague disease at early phase of Yersinia pestis cell cycle.

INTRODUCTION

Yersinia pestis (formerly *Pasteurella pestis*) is a Gram-negative rod-shaped bacterium belonging to the family Enterobacteriaceae (1). It is a facultative anaerobe that can infect humans and other animals and cause deadly disease "Plague" (2-4). It is also known as Biovar *Mediaevalis* which correspond to the Black Death (3). The complete genomic sequence is available for ten sub-species of *Y. pestis* including recently sequenced sub-species Z176003 which contains 3542 protiens (5,6). Currently, many of the medicine used for the treatment of Plague is not efficient therefore, we have always need to find new targets from the metabolism of ***Yersinia pestis*** which may act as new target for the drug development program against plague disease (7,8). In our research work, We determined the putative 3D structure of hypothetical protein (UPF0213 protein Y) from ***Yersinia pestis*** (http://pir.uniprot.org/uniprot/Q8ZBE1) via Comparative homology modeling tools. The technique of homology modeling with molecular docking have been already exploited to find alternative drug against pathogenic microorganism (9-23). Therefore, homology modeling is the pre step towards drug discovery for hypothetical pathogenic proteins.

MATERIALS AND METHODS

Input sequence

The protein sequence of hypothetical protein (UPF0213 protein Y) from ***Yersinia pestis*** (http://pir.uniprot.org/uniprot/Q8ZBE1) was obtained from uniprot and subjected to BLAST for sequence alignment.

Homology modeling

The protein sequence was subjected for comparative homology modeling via Swiss model (Arnold et al, 2006; Kiefer et al, 2009; Peitsch et al, 1995; Lorenza et al, 2008) and ESyPred3D (via Modeller 6v2) software's (Lambert et al, 2002) to generate putative 3D model. The model structure was further verified by PROCHECK and PROSA analysis.

Journal of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)

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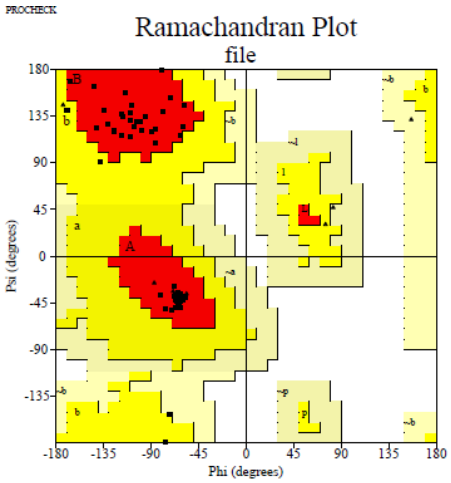
The model structure was further optimized by energy minimization via GROMOS96, implemented in Swiss pdb viewer software.

Functional site prediction

We subjected the model structure to different function and functional site prediction servers *e.g.* DALI, PROFUNC and Q-SITE FINDER. The BLAST and PSI-BLAST were used for function verification. On the other hand, the PROFUNC and Q-SITE FINDER were used for structure based functional site prediction.

RESULTS AND DISCUSSION

The BLAST, PSI BLAST (10 iteration), Phylogeny tree and UniPort analysis assigned putative Excinuclease ABC C subunit domain protein like function to UPF0213 protein Y. But no clue was finding about 3D structure of UPF0213 protein Y. Therefore, we used Swiss model and Modeller 6v2 (EsyPred3D) softwares for structure model determination. Both the softwares developed model based on the template pdb 1ZG2 (chain A) (with sequence Identity 38.30% (Modeller6v2), 39.50% (Swiss model)) and further verified by Procheck and ProSA servers. The RAMACHANDRAN PLOT analysis revealed that Modeller 6v2 (EsyPred3D) produced more accurate model as 100% of amino acid residues are in favoured (A, B, L) (Fig.1) and additional allowed (a, b, l, p) regions but in swiss model 98.70%.



Plot statistics

Residues in most favoured regions [A,B,L]	65	94.2%
Residues in additional allowed regions [a,b,l,p]	4	5.8%
Residues in generously allowed regions [~a,~b,~l,~p]	0	0.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	69	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	7	
Number of proline residues	0	
Total number of residues	78	

Fig.1 : Ramachandran plot analysis for EsyPred3D model.

ProSA verified that model by Modeller6v2 is in NMR region (known protein structures by NMR) with lower Z score of -3.22(Fig.2) but model by swiss model software failed to pass the ProSA criteria.

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Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)
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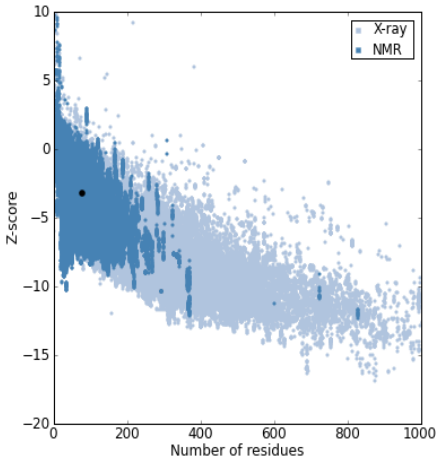

Fig.2: PROSA analysis.

The model via Modeller 6v2 was selected for further consideration (Fig.3). The energy minimization via GROMOS96 (implemented in Swiss pdb viewer) optimized the model from initial energy of -3071.358 KJ/mol to -3654.998 KJ/mol.

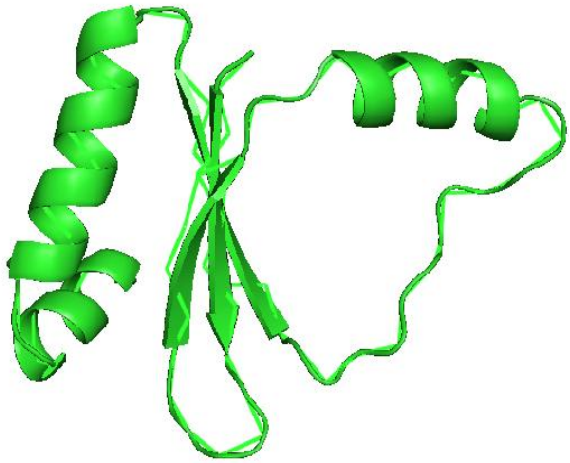

Fig.3: Model structure

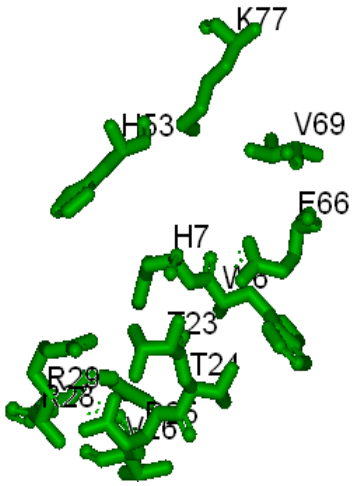

Fig.4: Functional site in model structure

DALI and PINTS was not able to produce any significant hit. But Q-site finder and Profunc detected putative functional site amino acid residues in UPF0213 protein Y. The Q-site finder detected W6, H7, T23, T24, D25, V26, R28, R29, H53 as putative active site residues(Fig.4) with site volume of 176 cubic Å with coordinates of binding box around selective site of Min Coords: (-9, -5, 5), Max Coords: (7, 8, 19). Profunc also predicted active site residues E66, V69, K77, RMSD 0.05 Å local sequence identity 50.00% in reverse template match. Therefore the structure prediction and functional site determination may provide putative information about functional role of UPF0213 protein Y and its amino acid residues. It may also help in structure based drug designing against ***Yersinia pestis*** as the predicted function of hypothetical protein UPF0213 protein Y i.e. Excinuclease may be in DNA repair mechanism of ***Yersinia pestis*** which may act as the potential target to prevent the Plague disease at early phase of ***Yersinia pestis*** cell cycle.

ACKNOWLEDGEMENTS

PK greatly acknowledges Indian Science and Technology Foundation (ISTF) for providing all financial supports and computer facility for running docking calculations.

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Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)

JOS

Nutritional importance of colostrum in different farm animals- A Critical Review

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a r t i c l e i n f o

Article history:

Received 1 August 2016
Revised 25 August 2016
Accepted 29 August 2016
Available online 31 August 2016

Keywords:

Colostrums,

Goats,

Cow,

Human

ABSTRACT

The nutritional importance of colostrum in different farm animals and it's quality has two basic facts that the colostrum quality begins to degrade immediately after calving and colostrum rapidly loses quality after about six hours post calving. Six hour colostrum has 5.3% albumin by weigh but 12 hour colostrum has 2.96% albumin. Colostrum serves mainly three functions are laxative, nutritive and protective. Laxative to aid in the certainty of the muconium lining of the digestive tract. Nutritive has provided an excellent energy source for the newborn. Energy reserves in the newborn are limited, and the high fat content of colostrum serves that purpose well. Protective contains antibodies (Igs) to protect the newborn kid until its own immune system begins functioning about 3 weeks of age. The newborn kid can absorb the essential antibodies in the colostrum only during its first 24 hours or so of life, as there is a rapid reduction in the permeability of the intestinal wall of the newborn to the antibodies. These antibodies protect the kid for the first 8-10 weeks against many diseases. It may be difficult to rear healthy, well-growing kids if they do not receive colostrum. Kids that do not receive colostrum will be less resistant to scours (diarrhea) and other aliments later in life.

INTRODUCTION

Colostrum is the first food for neonates after the parturition that provides them with all necessary nutrients. This is particularly important for the passive immunization of the newborn, as the combination of its various specific and non-specific antibacterial factors passes in the offspring and largely supports their protection against infections during the first days after birth (1, 2). The amount of colostrum first feeding should be equal to 4-5% of the kid's body weight. But during the first 24 hours the kid should receive an overall amount equivalent to 12-15% of its body weight (3).

If a kid is separated from the doe at birth, kid must be fed colostrum as soon as possible and for at least one day. If colostrum is not available from the kid's mother the kids may be fed with goat, ewe or cow colostrum that has been stored. The most suitable colostrum is from animals, which are starting

Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)
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their second or later lactation, as this colostrum contains higher levels of antibodies than does that from young females. Kids should receive colostrum 10% of their body weight within 24 hours. For example a 3kg kid should receive 300ml of colostrum within 24 hours. Usage Instructions of colostrum are activated in the mouth by saliva-via babes at breast, suckling calves and you and I. All mammals, including humans, at birth receive and benefit from colostrum. All newborn mammals receive colostrum orally, and not in an encapsulated form which bypasses the mouth, throat and pharynx, where vital biological processes occur. Immunological receptors in mouth and throat those are activated by colostrum and provide protection against invading microorganisms.

Mainly two type of colostrums**,** artificial and natural. The artificial colostrum feed is found widely distributed in calf and lamb management (4). According to some worker (5) Kids fed only with commercial colostrum do not acquire the necessary immunity to protect them during the first month of life.

Composition of ingredients in the artificial colostrum (Colostrum®, Vetoquinol) is gross protein 6.62%, fat (hydrolyzed) 22.50%, cellulose 0.15%, mineral matter 1.34%, sacarose 2.76%, ash 0.94%, total nitrogen 0.79%, calcium 0.14%, phosphorus 0.11%, sodium 0.1% and the humidity 32.88% when data supplied by the manufacturer. Mainly four type products are available in the market of artificial colostrums are lozenges, liquid powder and skin cream. Lozenges work as general purposes and Immune enhancement. Powder work as Body building, Athletics, Immunity and General Purposes. Liquid works as a stop nosebleed, rapidly healing crisis, skin injuries, eczema and other skin irritations. Skin cream work as moisturizes protects, accelerates the healthful replacement of old skin cells with new skin cells and restores skin elasticity and softness, eliminates blemishes.

The ingredients present in commercial colostrums are gross protein 8.95%, fat 8.66%, lactose 3.91% and IgG (mg/ml) 30.69. Composition of the RC (4°C) and FC (-20°C) fed (1:1) to kids twice per day for 2 days, each kid receiving 5% of the body weight per fed.

Physical nature

Colostrum is yellow to orange in color and thick and sticky. It is extremely easy to digest, and is therefore the perfect food to your baby. Human colostrum is a lemon yellow in the color while bovine colostrum is reddish yellow**.**

Chemical nature

Colostrum contains the following protecting, regulating and support factors.

A. Protection

Immunoglobulins, leukocytes, lectoferrin and lysozyme work as a protection. Immunoglobulins fight against bacteria, viruses and yeast. Colostrum contains immunoglobulis IgA, IgD, IgE and IgM. Leukocytes stimulate the production of interferon, which slows viral production. Lectoferrin defends against infection and

cancerous tumors it is also anti-inflammatory. Lysozyme destroys bacteria and virus.

B. Regulation

Proline-rich polypeptides, cytokines, interleukin-10 and lymphokines work as regulation in our body. Proline-rich polypeptides both initiate and suppress the immune response. Suppressing the immune system is required to prevent the immune system from attacking the body itself, as occurs with autoimmune diseases. Cytokines are interleukins that regulate the intensity and duration of the immune responses. They are highly anti-viral and anti-tumorous. Interlukin-10 reduces inflammation caused by arithritis, infection or injury, whether from surgery or trauma. Lymphokines regulate the immune response.

C. Support

Some supporting like as growth factors present in colostrum. Insulin-like growth factor-1 (IGF-1), Prolactin, Epithelial growth, Transforming growth factor A and B, Fibroblast growth factor, Gondotropin releasing hormones, Associated peptide and growth hormones etc. In the young human body produces maximum amounts of growth factors are produced. This is one of the reasons that the physical body and the mind age and why the supplementation of growth factors can slow reverse aging.

Growth factors

Promote wound healing and tissue repair, stimulate growth and regeneration of cartilage, nerves, bone and muscles, balance blood sugar, Increase the breakdown of fat, Serotonin uptake. It is believed that immunoglobulin factor-1 crosses the blood brain barrier with the result of increased mental acuity and increased serotonin uptake. Leptin increases the breakdown of fat and regulates metabolism and body weight. Nucleotides facilitate cellular energy transfer.

Dietary effects on colostrum nutrient content

A. Energy: Energy in colostrum is important to neonate immediately after birth. Calves are generally born with low energy stores. Colostrum fat therefore plays a major role in the supply of energy and in glucose homoeostasis of the neonate, and is critical to proper thermoregulation (6).

B. Protein: The colostral proteins are utilized by the neonate for protein synthesis and for the absorption of Ig. The availability of amino acids for protein synthesis and gluconeogenesis is important in establishing homeostasis in the neonate. Stimulation of protein metabolism post-birth requires large amounts of amino acids. A new born lamb is reported to synthesize 1.4g protein/h per kg of body weight (7).

Colostrum contains many proteins other than Ig. Both albumin and globulin are rapidly absorbed from the abomasums and are hydrolyzed to amino acids (7). Casein on the other hand accumulates in the abomasums and tends to be an important, although more slowly available, source of amino acids. Although Ig is more resistant to degradation, the large mass in colostrum makes this protein an important source of amino acids for the neonates.

Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)
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Table 1: The composition of goat's colostrums (8)

Ingredients	1st day	2nd day	3rd day
Specific gravity	1.039	1.035	1.034
Water	81.17	85.26	86.03
Total solids	18.83	14.74	13.97
Fat	8.21	5.15	4.64
Total Protein	5.69	4.21	3.60
Casein	3.14	3.18	2.84
Casein Globulin	4.90	3.58	3.19
Albumin	0.79	0.64	0.41
Globulin	1.76	0.40	0.35
Lactose	3.39	3.80	4.23
Ash	0.88	0.90	0.83

Mode of action

1. Age at first colostrum feeding

The intestinal epithelial cells lose their ability to absorb intact macromolecules after 24 hours because of the maturation of the cells and the development of the intracellular digestive apparatus (9), which begins shortly after birth. Hence there is a compelling reason to feed calves as soon as possible after birth to maximize the AEA and the acquisition of passive immunity. The establishment of microbial populations in the intestine, which may also be involved in reduced AEA after birth. The intestinal tract of the neonate is sterile at birth, but within a few hours environmental bacteria begin to colonize in the intestine. A one day old baby's stomach capacity is about 5-7ml.

Fig. 1: Infant stomach capacity

2. GIT effects

Effects on GIT development and function, and affects the digestive enzymes and gastrointestinal hormones (10, 11) besides the absorptive capacity (12). Trypsin inhibitor enzyme occurs naturally in colostrum (13). The high concentrations of growth factors in cow colostrum (mainly insulin like growth factors1 and 2--IGF-1 and IGF-2) and hormones (insulin) control the growth and development of GIT and contribute for the functional maturation of the organism during the first day after birth (1, 14, 15).

3. Micronutrients

The neonate is highly dependent upon colostral intake to obtain vitamin E after birth as α-tocopherol does not across the placenta in appreciable amounts. Moreover the vitamin E content of the colostrum is usually low unless the cow is provided supplemental dietary vitamin E (16). Many reports suggest that the α-tocopherol content of colostrum could be increased by supplementation of the cow's diet with vitamin E (16). Vitamin A and D also do not cross the placenta in significant amounts, so the calf must rely on the ingestion of colostrum for these vitamins as well (17). Selenium readily crosses the placenta and may

Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)
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accumulate in fetal tissues, particularly in liver (18).

4. Metabolic effects

Intake of colostrum brings about considerable metabolic changes in neonatal calves. The plasma concentrations of total protein and albumin arise as a consequence of the absorbed Ig. These changes are dependent on the timing and amounts of colostrum and IgG could not be altered when milk or milk replacer is fed in place of colostrums (19), Similarly, total and individual plasma EAA increased on day one of life after intake of colostrum for 3 days rather than only the first meal (20). Plasma urea levels in neonatal calves are dependent on intake, synthesis and degradation of protein (10).

5. Endocrinal effects

Ingestion of colostrum has marked effects on GI and pancreatic hormones. The cafes which failed to receive colostrum during the first 24 hours of life showed to have decreased plasma concentrations of gastrin and glucose-dependent insulinotropic polypeptide (10). Gluconeogenesis is essential to cover the requirements of glucose in neonates. Both glucagon and cortisol stimulate gluconeogenesis and are probably for glucose homeostasis in calves fed reduced amounts of colostrum (1). Pancreatic glucagon has antagonistic effects of insulin, hence its plasma concentrations in the neonatal calfes present an opposite picture to that of insulin.

Table 2: Contents of some hormones in colostrum & milk (21)

Hormones	Colostrum	Milk
Insulin	4.2-34.4 ng/mL	0.042-0.34 ng/mL
Total cortisol	4.4 ng/mL	0.35 ng/mL
Free cortisol	1.8 ng/mL	0.3 ng/mL
Prolactin	150 ng/mL	50 ng/mL
Progesterone	2.6 ng/mL	0.8 ng/mL

6. Antimicrobial Activity

Antibody-related antimicrobial activity of colostrum

Bovine colostrum contains a large number of naturally occurring antimicrobial substances (22, 23).

The biological function of cow's colostrum is not only to give nourishment to the offspring, but also to provide it with an immune protection against environmental pathogens. Cow colostrum has been used for a long time as feed supplements or substitutes for farm animals, mainly calves and piglets, in order to prevent contagious diseases (Diarrheal and other micro-bacterial diseases). The antibody augmented bactericidal activity of complement has been demonstrated in normal or immune colostrum and serum, but not against a great number of gram-negative bacteria, e.g. Aerobacter aerogenes (24), coliform (25), enterotoxigenic *E. coli* (26), Campylobacter jejuni (27) and Helicobacter pylori (28).

7. Effect on immunity

Colostrum is particularly important for the passive immunization of the newborn, as the combination of its various specific (immunoglobulins, Ig) and non-specific (humoral and cellular) antibacterial factors passes in the offspring and largely supports

their protection against infections during the first day after birth (1, 2). The intake of colostrum in the first hours after birth is extremely important for increasing the specific and non-specific resistance of calves against harmful pathogens, causing alimentary, respiratory and other disorders in the postnatal period (29).

Table 3: Colostrum composition of immunoglobulins in cow's colostrums (30, 21)

Immunoglobulins	Proportion in colostrum
IgG1	90% of all Ig
IgG2	1.6 to 6.4 mg/mL
IgM	5 to 8.7mg/mL
IgA	1.7 mg/mL
Albumin	1.2 to 2.66mg/mL
Transferrin	0.9 to 1.07mg/mL
Acute phase protein: α1-glycoprotein	1 to 1.65 mg/mL
Lactoferrin	1.2 to 2.6mg/mL
β-lactoglobulin	14mg/mL
A-lactoglobulin	4.5mg/mL

Function of Ig:

The intake of first colostrum occurs within 6 hours after calving, when Ig concentrations are the highest (29). The concentration of Ig and growth factors, are the highest in the first portions colostrum immediately after calving, and thereafter are rapidly decreasing (1, 29, 15). The Igs are produced by B lymphocytes. The immunological function mediated by the Igs depends on the Ig class. IgG antibodies have a multitude of functions, the most important of which is possibly the activation of complement-mediated bacteriolytic reactions. The kids did not present blood IgG at birth because the placenta structure in ruminants does not permit Ig transportation from mother to fetus. Another vital function of Igs is their ability to augment the recognition and phagocytosis of bacteria by leukocytes (opsonisation). Igs are also able to prevent the adhesion of microbes to surfaces, inhibit bacterial metabolism, agglutinate bacteria and neutralize toxins and viruses. IgM antibodies, although produced in smaller amounts than IgG, are considerably more efficient than IgG with regard to most of the above activities, especially to complement mediated lysis. IgA, in contrast, does not fix, complement or opposing bacteria, but agglutinates antigens, neutralizes viruses and bacterial toxins, and prevents the adhesion of entero-pathogen bacteria to mucosal epithelial cells.

In humans, is manifested by the passage of a considerable proportion of undigested active IgA from human colostrum through the gut of the neonate baby (31). In many *in vitro* studies shown that bovine IgG is also relatively resistant to proteolysis by digestive enzymes and is not inactivated by gastric acid (32). Some worker (33) demonstrated that about 19% of ingested IgG and IgM was found to retain immunological activity in the ileum of healthy human adults. Another worker (34) measured the survival of orally administered bovine Ig concentrate against Clostridium difficile toxins in the human GIT. The Igs of bovine colostrum provide the major antimicrobial protein against microbial infections and confer a passive immunity to the newborn calf until its own immune system

Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)
JOS

matures. Colostral Ig supplements designed for farm animals are commercially available in many countries (35).

Lactoferrin acts as an immune stimulating factor on the mucosal and the systemic immune system and that its binding to the mucosal cells is required for activation in animals (36). Breast-fed human infants ingest about 3g lactoferrin per day during the first week of life (37). A calf drinking 2 litres of colostrum ingested about 2g of lactoferrin per day. Lactoferrin is not easily digested by the enzymes in the intestinal tract and could be recovered from the feces of breast-fed infants with an intact iron-binding capacity can take place during the gastrointestinal passage via the iron-chelating mechanism.

8. Immunoglobulin growth factor

The cow a dry period between 40-90 days results in the best quality colostrum (38, 39). According to some worker (21) the physiological importance of growth factors in cow colostrum is IGF-1, IGF-2; colostric basic growth factor, proline rich polypeptide etc. The highest proportion of cow colostrum growth factors is insulin-like growth factors (IGFs). The concentration of IGF-1 in cow colostrums is (383-500µg/mL) but in women's colostrums is (18µg/mL) (40, 41).

Physiological effects of IGFs are their effects upon the trans-membrane, transport and metabolism of glucose, amino acids, nucleotides etc. Also, IGFs stimulate the synthesis and inhibit the breakdown of protein, DNA, RNA and regulate cell proliferation, differentiation in tissues, at the same time inhibiting apoptosis (programmed cell death) (42, 43, 15). The physiological importance of growth factors in cow colostrum is IGF-1, IGF-2; colostric basic growth factor, proline rich polypeptide etc. (21). IGF-1 is destroyed after heating milk to 121 C for 5 min. (44). Bovine colostrum is characterized by higher concentrations of insulin-like growth factor1 (IGF-1), IGF-11, insulin and prolactin and similar concentrations of glucagons but lower amounts of growth hormones (1).

Transforming growth factors (TGF)-TGF-α; TGF-β at lower concentration.their levels in colostrum (20-40mg/L) are significantly higher than in milk (1-2mg/L) (2).

Efficacy of antibody preparations in farm animals

Antibodies which block the H antigen on fimbriae of enteropathogenic *E. coli* strains have proven useful as a prophylactic measure (45). Some studies have provided evidence for the protective and therapeutic effect of Igs enriched from regular bovine cheese or colostrum from non-immunized cows against non-specific diarrheal diseases of newborn farm animals (46, 47) and humans (48).

Table 4: Efficacy of bovine milk against bacterial infections *in vivo*:

Farm animal	Target organism	Efficacy
Human infants	Enteropathogenic *E. coli*	Reduced *E. coli* in faeces (49)
Human Adults	*Helicobacter pylori*	No eradication or decrease in colonization of

Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)
JOS

		gastric antrum (50)
Human Adults	*Shigella flexneri*	Prevented shigellosis (51)
Human Adults	*Streptococcus mutans*	Reduced *S. mutans* level in dental plaque (52, 53)
Neonatal calves	Enterotoxigenic *E. coli*	Prevented diarrhoeal disease after experimental challenge (54)

In human, treatment of patients with AIDS, who receives 10g/d of normal bovine colostral Ig concentration up to 10 days (48). Some researcher used a similar preparation and dosage in HIV patients suffering from chronic diarrhea and reported significant clinical benefits with no side-effects for the duration of therapy. Purified Igs derived from colostrum from hyper-immunised cow, can provide protection against rotavirus in calves (55, 56).

Table 5: Efficacy of bovine immune milk against viral infections *in vivo*

Subject	Virus	Efficacy
Human infants	Poliomyelitis vaccine of Sabin type 2	Prevented infection of gastrointestinal tract (57)
Human infants	Rotavirus Wa1	Prevented infection (58)
Human infants	Human rotavirus MO	Prevented diarrhea (59)
New-born calves	Bovine rotavirus	Prevented infection
Neonatal calves	IgG from non-immunised cows, titres against RV proteins VP2, 4,6 and 7	Protected against diarrhea, no therapeutic effect on diarrhoea
New-born calves	Recombinant SA-11 (P2G3) rotavirus like IND (P/5/G6) particles	Prevented bovine rotavirus shedding and diarrhea (60)

Table 6: Efficacy of bovine immune milk against *Cryptosporidium* infections in Human:

Subject	Route of administration of immune milk	Efficacy
A three year old body	Via nasogastric tube	Vomiting and diarrhea removed in 5 days and oocysts absent from stools in 8 days (61)
AIDS patient	Direct duodenal infusion	Ceased diarrhea, stools formed, oocysts in stools absent (62)
Healthy human adult	Oral (10g of preparation three times a day	Reduced diarrhea and oocyst excretion after experimental challenge (63)

Evidence of the Anti-aging effect of Colostrum

Colostrum decreases in body fat, an increase in bone density and lean muscle mass, Thicker and more elastic skin changes.

The complement system

The complement system play a major role in the host defense mechanisms against infectious microbes, as it is involved both in specific and non-specific immunity. The antibodies absorbed from colostrum after birth, the complement system plays a crucial role in providing passive immunity to the newborn calf (64, 65). Several studies have demonstrated the occurrence of hemolytic or bactericidal complement activity in bovine colostrums (66, 28).

Human Transitional Milk

The change from colostrum to mature milk is a gradual process taking five days to weeks. The breast milk of women between the fifth and tenth day's after birth has been termed "Human transitional milk". In most species, the nitrogen partition is colostrum differ from that of later secretions with significantly more globulins and less casein that in mature milk. Therefore, colostrum is heated coagulable while milk is not.

Ruminant feed to improve health and colostrums

Many researchers, worked on herbal, probiotics and essential oils to alter digestive system to improve health via alteration of microbial ecosystem (67-78).

In other technique to optimize balance nutrients for milk production. Supplements of protein, starch and lipids to provide nutrients for milk production above those obtained when the efficiency of utilisation of the basal feed has been optimised. This way provides the nutrients in exactly the correct balance for additional milk production. By feeding a combination of non-protein nitrogen (NPN), minerals and by-pass protein. Once the efficiency of utilisation of the basal feed resource has been optimised, depends upon providing nutrients needed for the components of milk, e.g. the quantity and balance of glucose (for milk lactose), protein and fat, in a form that will by-pass the rumen.

CONCLUSION

Colostrum has a laxative effect on the baby, helping him pass his early stools, which aids in the excretion of excess bilirubin and helps prevent jaundice. Colostrum actually works as a natural and 100% safe vaccine. It contains large quantities of an IgA, which is a new substance to the newborn. IgA protects the baby in the place most likely to come under attack from germs, namely the mucous membranes in the throat, lungs, and intestines. Colostrum also contains high concentrations of leukocytes, protective white cells, which can destroy disease-causing bacteria and viruses.

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Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)

JOS

Effect of probiotic supplementation with different roughage: concentrate rations on *in vitro* rumen fermentation metabolites

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a r t i c l e i n f o

Article history:

Received 30 July 2016
Revised 9 August 2016
Accepted 19 August 2016
Available online 10 September 2016

Keywords:
Probiotics,
in vitro,
TVFA,
Goats

ABSTRACT

To evaluate the effect of probiotics (S. cerevisiae and Lactobacilli, single and combined) on in vitro total volatile fatty acids in Barbari goats. Supplementation of S. cerevisiae or lactobacilli alone or combination of both cultures had no effect on total VFA concentration, and the type of substrate with different proportion of R:C (40:60, 50:50 and 60:40) also had a similar effect on VFA concentration. However, combination of S. cerevisiae and lactobacilli (T_4) increased (P<0.001) propionate level with reduction of acetate due to such treatments. This resulted in a reduction (P<0.001) of the acetate/propionate ratio in T_4 as compared to other treatments. Since, the combination of yeast and lactobacilli has no added advantage in the present study over yeast alone.

INTRODUCTION

Alterations of rumen fermentation are common now a day, to improve animal growth and methane reduction (1, 2, 3). Probiotic effect is usually differs depending upon the probiotic dose level, diet composition, viable yeast cell number, strains of yeast, animal age and stage of growth (4). The main modes of action of yeast probiotics have Supplementation of growth factors to rumen micro-organisms, oxygen scavenging that creates more favorable conditions for the anaerobic communities and nutritional competition with autochthonous ruminal microbial species for energy (5, 6).

Various authors found that the yeast supplementation resulted in a numerical increase in concentration of ruminal ammonia-N (7). In contrast, in an *in vitro* study, Some workers reported that the *S. cerevisae* did not affect ruminal ammonia-N concentration in sheep fed the supplementation at 4g/d and 1.3g/kg of diet (8, 9), in lambs (10) and total-nitrogen in strained rumen fluid (10). Yeast culture feeding to lambs did not change the nitrogen intake, nitrogen voided in faeces, urine and balance (10).

During feed ingestion, some amounts of oxygen enter the rumen along with feed and it's adversely effect rumen environment as well growth of the rumen. Supplemented with yeast the volatile fatty acids were significantly (P<0.01) higher in sheep (11). Supplemented with *S. cerevisae*, the concentration of total VFA was significantly higher (12). Whenever, in lambs yeast culture did not affect the concentration of total VFA in strained rumen fluid (10). Some workers reported that supplementation of *S. cerevisiae* resulted in a quadratic increase of propionate, with a quadratic decrease of acetate: propionate under *in vitro* system (13). Similarly, enhanced production of propionate and reduction of acetate to propionate was observed due to DFM additions in either *in sacco* or *in vitro* (14,

Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)
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15). Presence of probiotics prevents ruminal acidosis by balancing the volatile fatty acids rations in the rumen (16). Other workers reported serum glucose is quite high in yeast based diet in ruminants (17), because probiotic induced the changes in the rumen fermentation process (16) and this change increase the propionate concentration in the rumen of lambs. However some study says supplementation of lactic acid bacteria (*Lactobacillus sporogens*) did not influence intake performance, higher growth, rumen fermentation pattern and blood chemistry. Similarly, other appetizer are not also valuable (18).

Since, the experiment has been formulated with the objective to evaluate the effect of supplementation of probiotics on rumen fermentation production under *in vitro* system. In the this experiment was conducted under controlled fermentation system in the laboratory for evaluation and screening of most promising probiotics culture or culture combination. Therefore, four treatments with control (no probiotics) = T_1, *Saccharomyces cerevisiae* (T_2, 125×10^5 cfu/0.5g substrate), *Lactobacilli* (T_3, 7.5×10^5 cfu/0.5 g substrate) and *Saccharomyces cerevisiae* (T_4, 62.5×10^5 cfu + *Lactobacilli* 3.75×10^5 cfu /0.5 g feed substrate were tested in this study with three substrate combinations (R:C=40:60 (D_1); 50:50 (D_2) and 60:40 (D_3)).

MATERIALS AND METHODS

The experiment was carried out at experimental unit of the Nutrition, Feed Resource and Products Technology Division, Central Institute for Research on Goats, Makhdoom, Farah, Mathura. The concentrate mixture were prepared with crude protein 18% and TDN 70%. Cluster bean hay was used as a roughage source during the *in vitro* trials. The experiment on *in vitro* rumen fermentation petterns were conducted under laboratory condition, two commercially available probiotics or direct fed microbial (DFM) were taken for the study of yeast culture (*Saccharomyces cerevisiae*) and lactic acid bacteria (*Lactobacilli*). The substrates were incubated in triplicate according to the procedure described by Tilley and Terry (19).

Sampling of feeds and chemical analysis

Estimating DM content the samples were ground with a Wiley mill in a laboratory and preserved in polythene bags for subsequent chemical analysis. Representative sample of concentrate and cluster bean hay used in the substrate was analysed presented in Table 1. The samples of feed offered of concentrate, cluster bean hay and its three substrate combination and residues left as faeces voided were analysed for crude protein, fat, Organic matter, Total carbohydrate and Total ash following the procedure of AOAC (20). Cell wall components in viz., neutral detergent fibre, acid detergent fibre, Hemi-cellulose, cellulose and lignin were estimated in accordance with Goering and Van Soest method (21).

Collection of rumen liquor and substrate

Rumen liquor was taken from the male Barbari goats maintained under uniform feeding system. Rumen liquor was collected from the donor goats by the stomach tube from all parts of the rumen into a clean thermo flask. The rumen liquor was taken to ensure the maintenance of optimum temperature while collecting and handling of rumen liquor.

in vitro techniques

In each *in vitro* bottle 0.5g (DM) of respective substrate was added. In each bottle 40 ml McDougall's buffer and 10 ml of SRL collected from donor animals by respective groups were added. Each

Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)
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bottle was infused with CO_2 before sealing with aluminum cap and rubber cork. The *in vitro* bottles were incubated for 48h at 39°C±0.5°C. The contents of the flask after 48h of incubation were filtered through Grade-1 crucible. The DM was estimated according to AOAC (20) in the samples of substrates as well as in the residues.

The pH of rumen fluid was determined within 10 min. of aspiration using digital pH meter, thereafter rumen fluid samples were strained through four layers of muslin cloth and frozen at -20ºC for further analysis. The strained rumen fluid (SRF) was used to determine total VFAs as per procedure (22). The Micro Kjeldahl procedure (21) was followed for total-N, ammonia-N**,** NPN and TCA-precipitable-N determination in the SRF.

Fractionation of VFAs in rumen fluid was done by GLC according to Erwin *et al.* (23). The supernatant was decanted and used for VFA fractionation in Amil Nucon Gas chromatography, series-5700 fitted with glass columns (chromosorb 101).

Statistical analysis

Data pertaining of probiotic supplementation under *in vitro* system with different roughage: concentrate ratios were statistically analyzed using with two-way ANOVA. The SPSS base 7.5 statistical package was used for statistical analysis.

RESULTS

Proximate and cell wall composition of feedstuffs were presented in Table 1. CP content was 18.98% in concentrate mixture and 16.85% in cluster bean hay. The Substrate used during the *in vitro* trial was prepared with varying levels of cluster bean hay and concentrate mixture.

The pH of the incubation medium was not affected by the supplementation of different combination of probiotics and substrates. The pooled value of pH ranged from 6.78 in T_2 and T_4 to 6.82 in T_1 (Table 2). However, there was a tendency of reduction of pH in the incubation medium of *in vitro* bottles when supplemented with *S. cerevisiae* alone (T_2) or in combination with *lactobacilli* (T_4). The interaction effect of treatment × substrate was also found similar.

The data on ammonia-N and other nitrogen fractions are presented in Table 2. Supplementation of the combined culture of *S. cerevisiae* plus *Lactobacilli* (T_4) resulted lower ($P<0.001$) total nitrogen in the incubation medium than other treatments and increased ($P<0.01$) NH_3-N concentration in the same treatment group. The pooled values of NH_3-N (mg/dl incubation medium) varied from 17.33 in T_2 to 21.53 in T_4. The substrate had also significant ($P<0.01$) impact on total-N and NH_3-N concentration, being highest in high concentrate based substrate D1 than D2 and D3. TCA-precipitable nitrogen remained unchanged due to probiotic supplementation under *in vitro* system, the values ranged from 17.62mg in T_1 to 18.55mg in T_2 (Table 2). High concentrate diet (R:C=60:40) resulted into higher levels of total-N and TCA-precipitable-N ($P<0.01$). The interaction effect of treatment and diet was significant for total-N and NH_3-N concentration in the incubation medium.

The data on total VFA concentration and its fractionations as influenced by treatments and types of substrates are presented in Table 3. Supplementation of *S. cerevisiae* or *lactobacilli* alone or combination of both cultures had no effect on total VFA concentration, and the type of substrate with different proportion of

Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)
JOS

R:C (40:60, 50:50 and 60:40) also a had similar effect on VFA concentration. The pooled values of total VFA (m mol/dl) varied from 10.24 in T_1 to 11.48 in T_2. Interaction effect (Treatment × Substrate) was also similar for total VFA. However, *S. cerevisiae* (T_2) or combination of *S. cerevisiae* and *lactobacilli* (T_4) increased ($P<0.001$) propionate level in the incubation medium and at the same time acetate level reduced significantly ($P<0.001$) due to such treatments. This resulted in a reduction ($P<0.001$) of acetate/propionate (A/P) ratio in T_2 and T_4 as compared to other two treatments. The values of A/P ratio ranged from 2.43 in T_4 to 3.20 in T_1. However, diet type and interaction of diet × treatment were found non-significant for acetate, propionate and A/P ratio.

Supplementation of *S. cerevisiae* or *lactobacilli* alone or combination of both cultures had no effect on total VFA concentration, and the type of substrate with different proportion of R: C (40:60, 50:50 and 60:40) also had a similar effect on VFA concentration. However, *S. cerevisiae* (T_2) or combination of *S. cerevisiae* and *lactobacilli* (T_4) increased ($P<0.001$) propionate level in the incubation medium and at the same time acetate level reduced significantly ($P<0.001$) due to such treatments. This resulted in a reduction ($P<0.001$) of acetate/propionate (A/P) ratio in T_2 and T_4 as compared to other two treatments.

DISCUSSION

A similar observation was reported by some worker (24, 25, 26) who observed little effect of supplementation on ruminal pH. Several studies have reported no change on ruminal pH, when supplemented with yeast in dairy cows (27), steers (28) and heifers (29) in latent acidosis. Ruminal pH numerically was increased, but no significant effects were observed when supplemented with yeast in cow (30). Whereas, supplementation of different additives shown various effects on ruminant diet in goats (31, 32 and 33). Therefore, multiple factors are responsible for positive effect of probiotics, specifically yeast culture, on stabilization of ruminal pH.

Some workers reported that *S. cerevisae* did not effect ruminal ammonia-N concentration in sheep fed the supplementation at 4g/d and 1.3g/kg of diet (8, 9), in lambs (10), in buffaloes (34) and total-nitrogen in strained rumen fluid (7). Some workers reported that in the presence of *S. cerevisae*, there was a decrease in ruminal ammonia concentration when lambs were 20 to 50 days old (35). Animals consuming yeast culture has a lower ruminal ammonia-N concentration and higher microbial protein synthesis in cow (38).

It has been revealed that yeast increased the ammonia-N content of culture fluid in cows (38). Yeast supplementation resulted in a numerical increase in concentration of ruminal ammonia-N (26, 4). Dietary supplementation with dry yeast culture (*S. cerevisiae*), improved the outflow rate of microbial nitrogen post ruminally in sheep (39).

Some workers have reported that the yeast supplementation generally tends to increase the ruminal VFA concentration, this increase being significant only, when the RFC-rich (Readily fermentable carbohydrates) ration is offered in restricted amounts (40, 41). While in our study yeast culture did not affect the concentration of total VFA in strained rumen fluid of lambs (10, 11). If yeast culture level increased in the diet, there was increased production of TVFA in the rumen fluid in calves (42) and in sheep (43).

The results of fractional VFA of the present study corroborated the findings of earlier workers, who have observed that there was a higher production of propionate and reduction of acetate to propionate ratio due to supplementation of yeast culture in lactating cows (44) and in steers (14). Similarly, enhanced production of propionate and reduction of acetate to propionate was observed due to DFM additions in either *in sacco* or *in vitro* (14, 15). Whereas, other workers reported that the presence of yeasts and oils have not modified the proportions of the different VFA in sheep (43, 45-47).

CONCLUSIONS

Supplementation of *Lactobacilli*, or *Saccharomyces cerevisiae* or combination of both cultures resulted similar effect on TVFA most of the fermentation metabolites in the incubation medium under *in vitro* system; however, propionate level was enhanced and acetate/propionate ratio was reduced due to the probiotics addition.

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Table 1. Composition (g/kg) of feeds used under *in vitro* system

Parameters	Concentrate mixture	Cluster bean hay	D1	D2	D3
Ingredient composition					
Linseed cake	30				
Barley grain	30				
Wheat bran	22				
Gram chuni	15				
Mineral mixture*	2				
Common salt	1				
Chemical composition (in DM)					
OM	913.9	917.9	915.5	915.9	916.3
CP	189.8	168.5	181.3	179.2	177.0
EE	48.6	31.5	41.7	40.0	38.3
Total carbohydrate	675.5	718.0	692.5	696.7	701.0
Ash	86.1	82.1	84.5	84.1	83.7
NDF	423.5	534.3	467.9	478.9	490.0
ADF	229.2	406.5	300.1	317.8	335.5
Cellulose	168.5	323.0	230.3	245.7	261.2
Hemi-cellulose	194.4	127.9	167.8	161.1	154.5
Lignin	49.1	73.9	59.0	61.5	64.0

*Vitamins + minerals feed supplement
Each kg Mineral mixture contains: Vitamin A, 625000 I.U.; Vitamin D3, 62500 I.U.; Vitamin E, 250 I.U.; Niacinamide, 1g; Calcium, 280g; Phosphorus, 120g; Cobalt, 0.2g; Copper, 1g; Iodine, 1g; Iron, 6g; Manganese, 1.2g; Selenium, 10.0g; Zinc, 2g.
D1, 40:60 (R: Conc.) = 40% Cluster bean hay and 60% concentrate mixture; D2, 50:50 (R: Conc.) = 50% Cluster bean hay and 50% concentrate
mixture; D3, 60:40 (R: Conc.) = 60% Cluster bean hay and 40% concentrate mixture.

Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)
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Table 2: Effect of probiotics supplementation with different roughage: concentrate rations on *in vitro* rumen fermentation.

Parameter	Diet	T_1	T_2	T_3	T_4	Pooled Mean ±SE	Treatment P-Value	Diet P-Value	Treat. ×Diet P-Value
pH	D1	6.89	6.75	6.83	6.85	6.83±0.03			
	D2	6.86	6.69	6.76	6.66	6.74±0.04			
	D3	6.72	6.88	6.86	6.84	6.82±0.03			
Pooled Mean ±SE		6.82± 0.04	6.78± 0.04	6.81± 0.03	6.78± 0.04	6.80±0.02	0.770	0.105	0.120
Total-N (mg/dl)	D1	53.20	56.00	52.50	52.85	53.64c±0.64			
	D2	50.05	50.40	50.40	43.40	48.56a±0.84			
	D3	54.60	51.45	51.45	50.05	51.89b±0.53			
Pooled Mean ±SE		52.62^b ±0.66	52.62^b ±0.83	51.45^b ±0.62	48.77^a ±1.31	51.36±0.26	0.000	0.000	0.001
TCA-ppt-N (mg/dl)	D1	18.90	21.70	18.20	19.25	19.51b±0.67			
	D2	17.85	18.20	18.20	16.80	17.76ab±0.52			
	D3	16.10	15.75	17.15	17.85	16.71a±0.79			
Pooled Mean ±SE		17.62 ±0.68	18.55 ±1.18	17.85 ±0.75	17.97 ±0.69	18.00±0.40	0.865	0.024	0.557
NPN (mg/dl)	D1	34.30	34.30	34.30	33.60	34.13a±0.46			
	D2	32.20	32.20	32.20	26.60	30.80a±0.85			
	D3	38.50	35.70	34.30	32.20	35.18b±0.96			
Pooled Mean ±SE		35.00^b ±1.06	34.07^b ±1.02	33.60^b ±0.49	30.80^a ±1.14	33.37±0.39	0.004	0.000	0.238
NH3-N (mg/dl)	D1	19.78	15.58	22.05	26.78	21.04b±1.44			
	D2	17.85	18.03	15.93	18.20	17.50a±0.48			
	D3	16.98	18.38	16.10	19.60	17.76a±0.51			
Pooled Mean ±SE		18.20^a ±0.84	17.33^a ±0.50	18.03^a ±1.48	21.53^b ±1.23	18.77±0.42	0.006	0.002	0.009

Means in the same row with the different superscripts (a, b and c) are significantly different.

*T_1 (Control), T_2 (*Saccharomyces cerevisiae*), T_3 (*Lactobacilli*) and T_4 (*Saccharomyces cerevisiae* with *Lactobacilli*).

* D1= 40:60 (R: Conc.), D2= 50:50 (R: Conc.) and D3= 60:40 (R: Conc.)

Journal Of Science, 2016, Vol. (2), Issue 1
Journal Homepage: www.isto-india.org/jsc
ISSN : 2395-535X (print), ISSN: 2395-5368 (Online)
JOS

Table 3: Effect of probiotics supplementation with different roughage: concentrate rations on *in vitro* total volatile fatty acids

parameter	Diet	T_1	T_2	T_3	T_4	Pooled Mean± SE	Treatment P-Value	Diet P-Value	Treat. × Diet P-Value
TVFA (m mol/dl)	D1	9.06	12.21	11.23	10.55	10.76±0.60			
	D2	10.50	10.75	11.31	10.56	10.78±0.72			
	D3	11.16	11.48	11.38	10.05	11.02±0.44			
Pooled Mean± SE		10.24± 0.79	11.48± 0.90	11.30 ±0.45	10.39 ±0.46	10.85±0.37	0.536	0952	0.904
Acetate (%)	D1	67.3	64.80	67.06	64.02	65.81±0.85			
	D2	68.02	66.26	66.57	61.52	65.59±0.85			
	D3	69.36	62.88	68.09	60.80	65.28±1.05			
Pooled Mean± SE		68.25^{c}± 0.72	64.64^{b} ±0.95	67.24^{c} ±0.58	62.11^{a} ±0.93	65.56±0.41	0.000	0.869	0.335
Propionate (%)	D1	22.33	24.08	22.23	26.07	23.68±0.72			
	D2	21.52	24.15	21.50	24.88	23.01±0.61			
	D3	20.63	26.02	20.83	26.91	23.60±0.86			
Pooled Mean± SE		21.49^{a}± 0.49	24.75^{b} ±0.55	21.52^{a} ±0.53	25.95^{b} ±0.88	23.43±0.33	0.000	0.661	0.596
Butyrate (%)	D1	10.31	11.13	10.72	9.91	10.52±0.68			
	D2	10.47	9.59	11.93	13.60	11.40±0.91			
	D3	10.01	11.10	11.08	12.29	11.12±0.50			
Pooled Mean± SE		10.26± 0.64	10.61± 0.88	11.24 ±0.75	11.93 ±0.98	11.01±0.43	0.538	0.695	0.740
Acetate/Propionate ratio	D1	3.04	2.70	3.03	2.52	2.82±0.10			
	D2	3.18	2.76	3.13	2.48	2.89±0.10			
	D3	3.38	2.43	3.28	2.28	2.84±0.14			
Pooled Mean± SE		3.20^{b}±0 .10	2.63^{a}±0 .08	3.15^{b}± 0.08	2.43^{a}± 0.09	2.85±0.04	0.000	0.836	0.295

Means in the same row with the different superscripts (a, b and c) are significantly different.
*T_1 (Control), T_2 (*Saccharomyces cerevisiae*), T_3 (*Lactobacilli*) and T_4 (*Saccharomyces cerevisiae* with *Lactobacilli*).

* D1= 40:60 (R: Conc.), D2= 50:50 (R: Conc.) and D3= 60:40 (R: Conc.)