Purification and production of Novel Antioxidative peptides from fermented goat milk

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INTRODUCTION

❑ **Goat milk has many benefits in context of higher digestibility, distinct alkalinity, higher buffering capacity and certain therapeutic in medicine and human nutrition (Park, 2009).**

❑ **Goat milk fermented with different LAB increase the biologically active peptides from corresponding sequences of the precursor protein.**

❑ **Food-derived peptides has been considered as natural antioxidants. So, More food protein hydrolysates and antioxidant peptides have been found to exhibit antioxidant activity (Samaranayaka and Li-Chan, 2011).**

❑ **In this study, Surti (Indian breed) goat milk and *Lactobacillus fermentum* culture were used for producing peptides and its analysed through LC/MS and searched against many BIOPEP software for confirming Antioxidant peptides.**

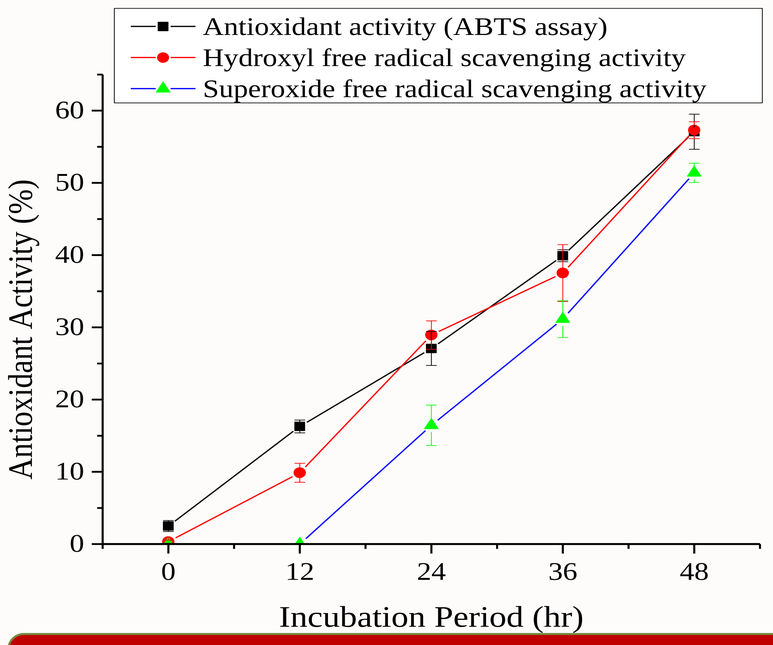
MATERIALS AND METHODS

- **Surti breed Goat milk procured from Instructional Livestock Farm Complex (ILFC), Veterinary College and *Lactobacillus fermentum* (M2) culture was collected from Culture Collection of Dairy Microbiology Department, SMC College of Dairy Science, Anand Agricultural University, Anand.**
- **Goat milk ferment with M2 culture (@ 2%) with different incubation periods (0, 12, 24, 36 and 48 h) at 37°C. Then, each sample centrifuged (14000 rpm at 4°C) and collected supernatant was tested with different antioxidant activities like, ABTS free radical scavenging activity (Hati *et al.*, 2013), Hydroxyl free radical scavenging activity (Li *et al.*, 2008) and Superoxide free radical scavenging activity (Liu *et al.*, 2010).**
- **Growth conditions (rate of inoculation 1.0, 1.5 & 2.0% and incubation periods 0, 6, 12, 24, 36 and 48 h) of the M2 culture was optimized by measuring the peptide content through O-phthalaldehyde (OPA) method (Hati *et al.*, 2015).**
- **In first Technique, 12% separating gel was used for SDS-PAGE analysis (Laemmli, 1970 and Carrasco-Castilla *et al.*, 2012). Two dimensional gel electrophoresis was carried out for purification of the peptides from water soluble extracts of fermented goat milk (Yang *et al.*, 2014) and In-Gel trypsin digestion was done.**
- **In second technique, 3 kDa and 10 kDa (Retentate as well as Permeate) supernatant of fermented goat milk sample injected into RP-HPLC for fractionation and also checked for antioxidant activities.**
- **After that, all the samples from HPLC and 2D-gel electrophoresis injected into RPLC/MS for characterization of peptides (Parmar *et al.*, 2018)**
- **Then, all generated spectra from RPLC/MS searched in MASCOT and mMass software as well as in PIR (Protein Information Resources) and Swissprot for confirmation with goat milk protein. Obtained peptides sequences were matched with antioxidant peptide database i.e. BIOPEP for confirming the antioxidant activity of fermented goat milk.**

RESULTS & DISCUSSION

Sr. No	Culture Name	Source of Isolation	Selective Media	Growth Conditions	Gene bank accession no.
1	*Lactobacillus fermentum* (M2)	Rice beverage	MRS Agar	37°C for 24h	MF951094

Antioxidant activities (ABTS assay, Hydroxyl free radical scavenging assay and Superoxide free radical scavenging assay) of fermented goat milk produced by M2 at different incubation periods

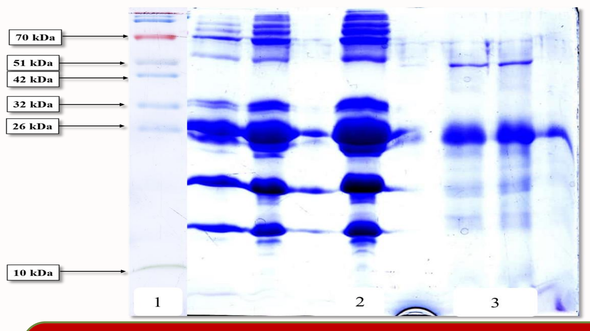


Effect of inoculation rates and incubation periods on proteolytic activity (mg/ml) of M2 in goat milk

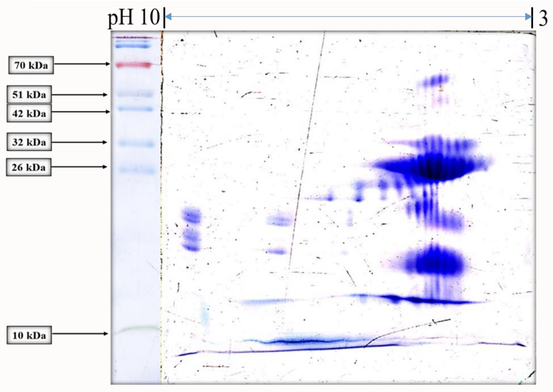
Rate of Inoculum	0 h	6 h	12 h	24 h	36 h	48 h
1.0 %	1.73±0.05^{a}	2.42±0.35^{b}	3.32±0.15^{c}	4.81±0.20^{e}	5.70±0.05^{f}	7.04±0.24^{h}
1.5 %	1.63±0.32^{a}	2.66±0.14^{b}	3.59±0.09^{cd}	5.32±0.13^{f}	6.13±0.44^{g}	7.34±0.03^{h}
2.0 %	1.66±0.20^{a}	2.77±0.02^{b}	3.76±0.07^{d}	5.65±0.16^{f}	6.53±0.64^{g}	8.13±0.16^{i}

Protein profile of goat milk fermented by M2 revealed by SDS-PAGE

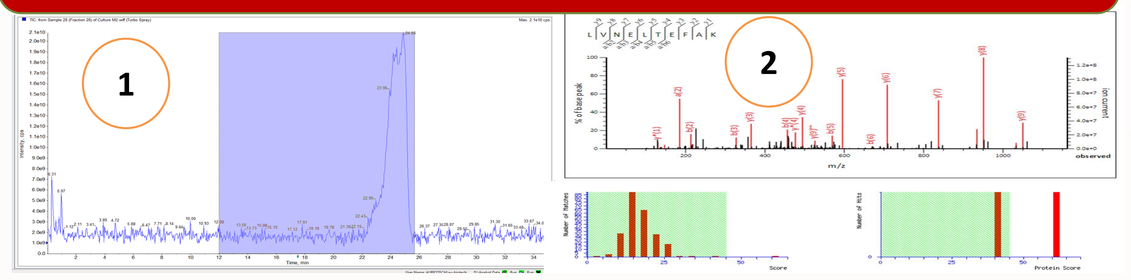
(1: Protein ladder, 2: unfermented goat milk and 3: fermented goat milk)

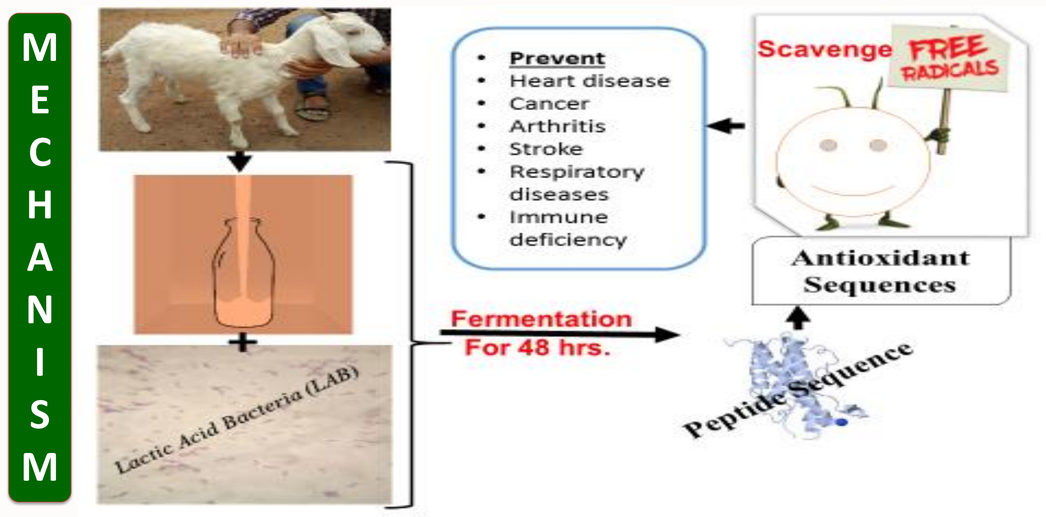


2D Gel Electrophoresis of goat milk fermented by M2



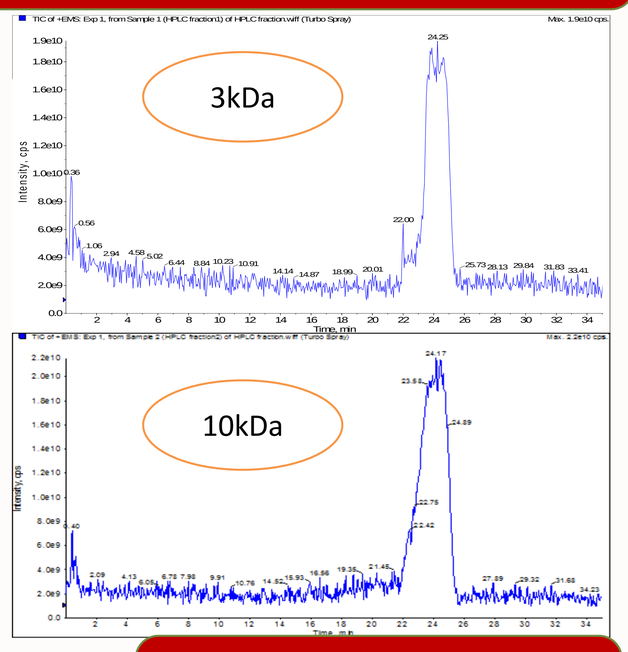
1) The Total ion chromatogram of 2D-spot (EMS to EPI scan in LC-MS) and 2) MS/MS spectrum of fraction inspected in MASCOT database identified as LVNELTEFAK for M2 culture and its protein score





- In fractionation with RP-HPLC, 3 kDa retentate shown highest antioxidant activity (ABTS assay), hydroxyl free radical scavenging activity and superoxide free radical scavenging activity i.e. 52.76, 29.68 and 43.10 %, respectively.
- The highest relative proteolytic activity was shown by 10 kDa permeate i.e. 60.79 %.

The Total ion chromatogram of HPLC fraction produced by M2 culture (3 and 10 kDa Permeate) (EMS to EPI scan in LC-MS)



All sequences found in 2D-PAGE analysis of fermentation of goat milk by using M2

2D-PAGE Sequence
LVNELTEFAK
SCQDQPTTLAR
RALAPAGAPGR
SPAQTLQWQVLPNTVPAK
VYVEELKPTPEGNLEILLQK
KVMAAAGGSTLK
KPSDDEESR
AAEHLSSIMK
AGVNDIK

All sequences found in 3 and 10 kDa permeate of HPLC fraction of fermentation of goat milk by using M2

HPLC fraction	Sequence
3 kDa Permeate	EDVPSER
	TIDMESTEVFTKK
	ETMVPKHK
10 kDa Permeate	YIQKEDVPSER
	FFIFTCLLAVALAK
	LAFNPTQLEGQCHV

CONCLUSIONS

- Goat milk fermented with *Lactobacillus fermentum* (M2) exhibited notable antioxidant activities. The highest antioxidant activity (ABTS assay) (57.09%), hydroxyl free radical scavenging activity (57.30%) and superoxide free radical scavenging activity (51.40%) found after 48h at 37oC by M2 culture and proteolytic activity was maximum (8.13 mg/ml) at 2% inoculation rate after 48h of incubation.
- In SDS-PAGE and 2D-PAGE analysis, 10-51 kDa protein bands were observed from fermented goat milk. RALAPAGAPGR and VYVEELKPTPEGNLEILLQK peptides sequence from 2D-PAGE were matched with antioxidant fraction of ALAPAG (β-lactoglobulin) and YVEEL (β-lactoglobulin) on BIOPEP databases, respectively.
- TIDMESTEVFTKK and FFIFTCLLAVALAK peptide sequences from various HPLC fractions (3kDa permeate and 10kDa permeate, respectively) were also matched with antioxidant fraction of EEEKNRLTKKTKLT (α-casein) and SALAM (β-lactoglobulin) on BIOPEP databases, respectively.
- Further, *in vivo* study is required to validate the health claim, particularly antioxidant activity on small animal or human subjects.