EFFECT OF PROCESSING ON FORMATION AND DEGRADATION OF BCM 7 IN MILK AND SELECTED MILK PRODUCTS

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

DAIRY CHEMISTRY

BY

SULAXANA KUMARI

M.Sc. (Food Science and Technology)

DAIRY CHEMISTRY DIVISION

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE

(DEEMEDUNIVERSITY)

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APPROVED BY :

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This is to certify that the thesis entitled "**EFFECT OF PROCESSING ON FORMATION AND DEGRADATION OF BCM 7 IN MILK AND SELECTED MILK PRODUCTS"** submitted by **SULAXANA KUMARI** in partial fulfilment of the requirement for award of the degree of **DOCTOR OF PHILOSOPHY** in **DAIRY CHEMISTRY** of the ICAR-National Dairy Research Institute (Deemed University), Karnal (Haryana) is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Date: 23/9/21

Dr. (Mrs) Bimlesh Mann
MAJOR ADVISOR

DEDICATEDTO HUSBAND

MOTHER IN-LAW,

SON-KANISHKA SINGH

&

DAUGHTER-SHAMBHAVI SINGH

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ABSTRACT

The present study was conducted to standardize the analytical method for the quantification of BCM 7 in milk and milk products and to evaluate generation and degradation of BCM 7 during processing of milk and milk products. RP HPLC method was standardized for the quantification of BCM 7 in milk and milk products. Calibration standards were prepared in milk matrix system and the linearity was observed in range of 5.0-125.0 μg/ ml with R^2 value 0.98. The limit of detection (LOD) and limit of quantification (LOQ) were 6.06 and 20.20 μg/ml respectively. To evaluate the generation and degradation of BCM 7 during different processing condition, selected milk and milk products were prepared with milk obtained from A1A1 and A2A2 genotype cow. For procurement of pure genotype milk 'KARAN FRIES' (Tharparkar X Holstein Friesian) bovine breed was selected. LTLT pasteurization, boiling and sterilization heat treatment were selected to evaluate the effect of heating on release of BCM 7 in processed milk and their digestive extracts. BCM 7 was not identified in raw, pasteurized, boiled and sterilized milk prepared from A1and A2 milk. BCM 7 was not detected in salivary and gastric extracts of raw and all types of heat processed milk. However BCM 7 was detected after the simulated *in-vitro* intestinal digestion of all milk samples. Concentration of BCM 7 in milk of A1A1 genotype cow was approximately 10-12 times higher compare to milk from A2A2 genotype cow irrespective to type of heat processing. As heat intensity increased content of BCM 7 decreased in gastrointestinal extracts of milk from both genotype cows. About 52 %, 77 % and 84 % reduction in BCM 7 content were observed by pasteurization, boiling and sterilization treatments respectively. Quantification of BCM 7 in gastrointestinal digestive extracts of raw and processed milk by ELISA assay showed lower concentration of peptide but similar trend as evaluated by RP-HPLC method. LCMS/MS peptide sequence analysis of intestinal digestive extracts of raw milk showed that BCM 7 (60-66), and BCM 7 like peptides such as β (59-66), β (53-66), β (56-66) were released from A1 milk. However release of BCM 7 did not detected in A2 milk but BCM 7 like peptides such as β (59-66), β (58-66) β (59-68), β (58-68), β (57-68), β (56-68), β (55-68) were identified.

Effect of fermentation and storage on generation and degradation of BCM 7 was studied in *dahi* prepared with A1 and A2 milk. BCM 7 did not identified in both types of *dahi* during storage up to 6 days at refrigerated temperature. Release of BCM 7 or BCM 7 like peptides were observed in gastric digestion of *dahi* prepared from A1 and A2 milk at very low concentration. In A2 *dahi* BCM 7 or BCM7 like peptides were detected up to 2 days of storage. However gastric extracts of A1 *dahi* showed the release of BCM 7 throughout the storage period. Release of BCM 7 and BCM 7 like peptides was identified in all samples of *dahi* after simulated *in-vitro* intestinal digestion. The content of BCM 7 and BCM 7

like peptides in A1 *dahi* was approximately 10 time higher compare to A2 *dahi*. Fermentation process has degradation effect on release of BCM 7 during storage and concentration of such peptides decreased with time at low temperature irrespective to the type of *dahi*. When digestive extracts of *dahi* were analyzed for quantification of BCM 7 by ELISA assay showed similar results but in low concentration compare to HPLC results were significantly higher than the results obtained by ELISA assay. Although, the trend of release of BCM 7 in *dahi and their digestive extracts* was similar in both assays. Generation and degradation of BCM 7 was studied during ripening of Cheddar cheese prepared from A1 and A2 milk. Initially BCM 7 was not identified in both type of Cheddar cheese. After four months of ripening presence of BCM 7 was identified in A1 type Cheddar cheese. In A2 Cheddar cheese BCM 7 and BCM 7 like peptides were identified after seven months of ripening. Release of BCM 7 and BCM 7 like peptides in gastric extracts of A1 cheese and A2 cheese were observed after 1 month and 2 months of ripening respectively. Concentrations of such peptides were higher in gastric extracts of A1 Cheddar cheese compare to A2 cheese. All samples of Cheddar cheese showed the release of BCM 7 and BCM 7 like peptides during intestinal digestion. The released of BCM 7 and BCM 7 like peptides were approximately 10 times higher in A1 than A2 Cheddar cheese. On ripening concentration of such peptides content decreased with time. Quantification of BCM 7 in digestive extracts of Cheddarcheese by ELISA assay was lower than the HPLC results. Acid and heat coagulated *Chhana* prepared from milk of A2A2 and A1A1 genotype cow. BCM 7 was not identified in all undigested, salivary and gastric digestive extracts of A2 and A1 *Chhana*. However released of BCM 7 and BCM 7 like peptide were identified in all fresh and stored samples of both genotypes. The concentrations of BCM 7 and BCM 7 like peptides were 2.5 times higher in A1 *Chhana* than A2 *Chhana*. Concentrations of these peptides decreased with storage. Results obtained by ELISA were in inline with the RP-HPLC findings. From present study it is concluded that generally BCM 7 released during intestinal digestion of milk and milk products. Release of BCM 7 is specific to milk containing A1 variant of β-CN, but a number of similar types of peptides were generated during intestinal digestion in milk and milk products having both variants of β-CN. Different processing techniques such as thermal degradation, fermentation, storage and ripening has significant effect on thedegradation of BCM 7.

<u>सार</u>

वर्तमान अध्ययन दूध और दूध उत्पादों में BCM 7 की मात्रा का मानकीकरण करने और दूध और दूध उत्पादों के प्रसंस्करण के दौरान BCM 7 के उत्पादन और क्षरण का मूल्यांकन करने के लिए आयोजित किया गया था। दूध और दुग्ध उत्पादों में BCM 7 की मात्रा का निर्धारण करने के लिए RP-HPLC पद्धति को मानकीकृत किया गया था। दूध मैट्रिक्स प्रणाली में अंशांकन मानक तैयार किए गए थे और रैखिकता 5-125μg / ml की सीमा में R^2 मान 0.98 के साथ देखी गई थी। BCM 7 का निष्कर्षण 30-40 मिनट के लिए 4^0 c पर 4500 rpm पर सेंट्रीफ्यूजेशन द्वारा किया गया था, पीएच समायोजन 4.6 और पानी में घुलनशील पेप्टाइड अंश प्राप्त करने के लिए बार-बार सेंट्रीफ्यूजेशन, इसके बाद 0.45 μ झिल्ली फिल्टर द्वारा निस्पंदन किया गया था। पता लगाने की सीमा (LOD) और परिमाणीकरण की सीमा (LOQ) क्रमशः 6.06 और 20.20 μ g/ml थी। दूध से निष्कर्षित BCM 7 ने अपने जलीय घोल की तुलना में 35-45% कम प्रतिक्रिया दिखाई, जिसे मैट्रिक्स प्रभाव कहा जाता है। विभिन्न प्रसंस्करण स्थितियों के दौरान BCM 7 के उत्पादन और क्षरण का मूल्यांकन करने के लिए, A1 और A2 दूध में चयनित दूध और दूध उत्पाद तैयार किए गए थे। जीनोमिक डेटा के आधार पर विभिन्न जीनोटाइप के स्वस्थ जानवरों का चयन किया गया था। थर्मल प्रोसेसिंग के प्रभाव का अध्ययन करने के लिए तीन प्रसंस्करण स्थितियों का चयन किया गया: LTLT पास्चुरीकरण, उबालने और स्टरलाइज़। प्रसंस्कृत दूध के पाचन क्षमता पैटर्न का मूल्यांकन करने के लिए नकली इन-विट्रो डाइजेस्टिबिलिटी प्रोटोकॉल (SGID) का उपयोग किया गया था। RP-HPLC विश्लेषण पर यह देखा गया कि A1 और A2 दूध और उनके लार के साथ-साथ गैस्ट्रिक अर्क से तैयार कच्चा, पास्चुरीकृत, उबला हुआ और स्टरलाइज़ दूध BCM 7 की उपस्थिति नहीं दिखाता । BCM 7 की उपस्थिति नकली इन-विट्रो आंतों के अर्क में की गई थी। सभी दूध के नमूनों में A1 दूध में BCM 7 की सांद्रता A2 दूध की तुलना में 10-12 गुना अधिक थी। जैसे-जैसे गर्मी की तीव्रता बढ़ी, BCM 7 की मात्रा कम होती गई। पाश्चराइजेशन, उबालने और स्टरलाइज़ करने पर BCM 7 की मात्रा में क्रमशः 52%, 77% और 84% की कमी आई। RP-HPLC द्वारा प्राप्त परिणामों को प्रतिस्पर्धी ELISA परख का उपयोग करके मान्य किया गया था। यह देखा गया कि विभिन्न प्रसंस्कृत दूध में BCM 7 की उपस्थिति RP-HPLC परिणामों के समान थी । लेकिन ELISA ने BCM 7 की कम सांद्रता दिखाई। RP-HPLC परिणामों को LCMS विश्लेषण द्वारा और अधिक प्रमाणित किया गया। कच्चे A2 और A1 दूध के BCM 7 (RT के 2 मिनट) के Lyophilized नकली इन-विट्रो गैस्ट्रो-आंत्र और RP-HPLC अंशों का ESI-QUAD-TOF का उपयोग करके विश्लेषण किया गया था। BCM 7 (60-66), और BCM 7 जैसे पेप्टाइड्स जैसे β(59-66), β (53-66), β (54-66) β (59-68), β (58-68), β (57-68), β (56-68) and β (55-68) के कच्चे दूध के इन विट्रो सिम्युलेटेड आंतों के पाचन के दौरान A1 दूध में देखा गया था। जहाँ A2 दूध में BCM 7 की पहचान नहीं हुई लेकिन BCM 7 जैसे पेप्टाइड्स जैसे β (59-66), β (58-66) β (59-68), β (58-68), β (57-68), β(56-68), β(55-68) देखे गए।

A2 और A1 दूध से तैयार दही में BCM 7 के उत्पादन और क्षरण पर किण्वन और भंडारण के प्रभाव का अध्ययन किया गया । A2 और A1 दूध से तैयार दही में रेफ्रिजरेटेड तापमान पर 6 दिनों तक रखने पर भी BCM 7 की उपस्थिति को नहीं दिखाया । A2 दूध और A1 दूध से तैयार दही के गैस्ट्रिक अर्क ने बहुत कम सांद्रता में BCM 7 या BCM 7 जैसे पेप्टाइड्स की उपस्थिति को दिखाया। A2 दही के नमूनों में BCM 7 या BCM 7 जैसे पेप्टाइड्स की उपस्थिति को 2 दिनों के भंडारण तक दिखाया । जबकि A1 दूध से तैयार दही के गैस्ट्रिक अर्क ने कम सांद्रता पर भंडारण अवधि के दौरान BCM 7 की उपस्थिति को दिखाया। दही के नकली इन-विट्रो आंतों के पाचन ने दोनों प्रकार के जीनोटाइप में BCM 7 और BCM 7 जैसे पेप्टाइड्स की उपस्थिति को दिखाया और A1 दही में उनकी सांद्रता A2 दही की तुलना में 10 गुना अधिक थी। इसके अलावा दही के नमूनों के आंतों के अर्क में BCM 7 की सांद्रता भंडारण के साथ कम हो गई। RP-HPLC द्वारा प्राप्त परिणामों की ELISA से तुलना करने पर, यह देखा गया कि RP-HPLC के परिणाम ELISA परख द्वारा प्राप्त परिणामों की तुलना में काफी अधिक थे। हालाँकि, स्वरूप दोनों परख में समान थे। A2 और A1 दूध से तैयार किए गए चेडर चीज़ के पकने के दौरान BCM 7 के उत्पादन और क्षरण का अध्ययन किया गया । प्रारंभ में दोनों प्रकार के चेडर चीज़ में BCM 7 की उपस्थिति नहीं देखी गई । पकने के चार महीने बाद A1 प्रकार के चेडर चीज़ में BCM 7 की उपस्थिति देखी गई । A2 चेडर चीज़ में BCM 7 और BCM 7 जैसे पेप्टाइड्स की उपस्थिति पकने के सात महीने बाद देखी गई । A1 और A2 चीज़ के गैस्ट्रिक अर्क में BCM 7 की उपस्थिति क्रमशः 1 महीने और 2 महीने के पकने के बाद देखी गई। A2 चीज़ की तुलना में A1 चेडर चीज़ के गैस्ट्रिक अर्क में BCM 7 की सांद्रता अधिक थी। चेडर चीज़ के सभी नमूनों में आंतों के पाचन के दौरान BCM 7 की उपस्थिति को दिखाया। BCM 7 की सांद्रता A2 चीज़ की तुलना में A1 चीज़ में लगभग 10 गुना अधिक थी और BCM 7 की सांद्रता पकने के समय के साथ कम हो गई थी। ELISA परख में प्राप्त परिणाम RP-HPLC परिणामों से कम थे। BCM 7 पर अम्ल वर्षा या जमावट के प्रभाव का अध्ययन करने के लिए A2 और A1 दूध के साथ छेना तैयार किया गया था। सभी छेना नमूनों और उनके लार और गैस्ट्रिक पाचन अर्क में BCM 7 की उपस्थिति नहीं थी। आंतों के पाचन के दौरान ताजा तैयार किए गए A2 और A1 छेना में और 6 दिनों तक संग्रहीत नमूनों में BCM 7 की उपस्थिति देखी गई थी। A1 में BCM 7 की सांद्रता A2 छेना की तुलना में 2-2.5 गुना अधिक थी और भंडारण अवधि के साथ BCM 7 सांद्रता कम हो गई। RP-HPLC द्वारा प्राप्त परिणाम ELISA निष्कर्षों के समान थे।

वर्तमान अध्ययन से यह निष्कर्ष निकाला गया है कि आम तौर पर दूध और दूध उत्पादों के आंतों के पाचन के दौरान BCM 7 निर्मित होता है। जैसा कि MS विश्लेषण से संकेत मिलता है कि आंतों के पाचन के दौरान BCM 7 का विमोचन A1A1 प्रकार के लिए विशिष्ट है। विभिन्न प्रसंस्करण स्थिति जैसे थर्मल, किण्वन, भंडारण और पकने का BCM 7 के क्षरण पर महत्वपूर्ण प्रभाव पड़ता है।

LIST OF ABBREVIATIONS

0	Degree Celsius
AR	Analytical Reagent
BCM	Beta Casomorphine
β-CN	Beta Casein
Bt-tracer	Biotinylated tracer
COB	Clot on Boiling
DAD	Diod Array Detectotor
DH	Degree of Hydrolysis
ELISA	Enzyme Linked Immunosorbent Assay
EIA	Enzyme Immuno Assay
EFSA	European Food Safety Authority
f	Fraction
g	Gram
HCl	Hydrochloric Acid
IDL	Instrumental Detection Limit
IQL	Instrumental Quantification Limit
KF	Karan Fries
LAB	Lactic Acid Bacteria
LOD	Limit of Detection
LOQ	Limit of Quantification
LTLT	Low Temperature Long Time
pH	Potential of Hydrogen
µl	Micro liter
M	Molar
µg	Microgram
mg	Milligram
ml	Milliliter
mM	Millimolar
ng	Nano gram
NBAGR	National Bureau of Animal Genetic Resources

nm	Nanometer
OD	Optical Density
PepX	X-prolyl-dipeptidyl-aminopeptidase
RT	Retention Time
RP-HPLC	Reverse Phase High Pressure Chromatography
RPM	Revolutions Per Minute
SA-HRP	Steptavidin-conjugated Horseradish Peroxidase
SD	Standard Deviation
RSD	Relative Standard Deviation
TCA	Tri chloro acetic acid
TFA	Tri flouro acetic acid
TNBS	2, 4, 6 Trinitrobenzenesulfonic acid
UHT	Ultra Heat Treatment
UV-Vis	Ultraviolet- Visible Spectroscopy

Amino acid single and three letter codes		
Name of AminoAcid	**Three letter code**	**One letter code**
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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CHAPTER –1

Introduction

In recent years superiority of milk of A2A2 genotype bovine over A1A1 genotype is gaining special attention due to various health implications such as human chronic diseases, diabetic, heart diseases, neurological disorder-autism, schizophrenia and sudden infant death syndrome etc, seemed to be linked with consumption of milk from A1A1 genotype cattle (Thiruvengadam *et al*., 2021). Potential factors derived from milk that contribute to these disorders are β-casein (β-CN) derived peptides known as beta Casomorphins (BCMs). These are the groups of peptides, having morphine-like characteristics. As such BCM peptides are encrypted in an inactive form in β-CN and released by various dairy processing conditions such as fermentation, heat processing or storage of dairy products (Brooke-Taylor *et al*., 2017). These peptides consist of 4 to 11 amino acids residues and having similar sequence of first three amino acids. β-CN accounts for 39 % of total casein, comprises of 209 amino acid residue sequence and exists in 15 different genetic variants (Gallinat *et al*., 2013). Each variant differs from other in amino acid substitution at fixed position. Classification of A1 or A2 milk is based on the type of β-CN variant present in the milk. Among all variants A1 and A2 of β-CN are most predominant in bovine milk in India (Mishra *et al*., 2009). β-CN A2 is the original variant which has proline at position 67 and substituted by histidine in A1 variant at same position. (Daniel *et al*., 1998). Milk of different species such as sheep, goat, buffalo, pig, horse, donkey and human milk carries proline at 67 position and similar to A2 type variant. Milk consisting either A1 or A2 variant are known as A1 milk or A2 milk, respectively. In A1 variant of β-CN peptide bond between isoleucine and histidine (66-67) is readily cleavage by proteases to the seven amino acid sequence (Try-Pro-Phe-Pro-Gy-Pro-Ile) known as BCM 7, whereas in A2 variant peptide bond between isoleucine-proline are resistant to enzymatic cleavage and hence formation of BCM7 does not occur (Jinsmaa and Yoshikawa 1999). BCM 7 is most extensively studied peptide for its opioid characteristics and responsible for various health issues (Thiruvengadam *et al*., 2021). However European Food Safety Authority (2009) published a report and concluded that studies performed to date did not allow a conclusion on the role of BCM 7 or A1 β-CN as a causative factor for various health problems. As such BCM peptides are encrypted in an inactive form in parent caseins and may be released by various dairy processing conditions such as heat processing, fermentation, ripening or storage of dairy products (Choi *et al*., 2012). In recent years, a number of studies reported the presence of BCM 7 in milk and milk products (Cieślińska *et al*., 2007, Cieślińska *et al*., 2012; Nguyen *et al*., 2014; Nguyen *et al*., 2015, Asledottir *et al*., 2017, Miguel *et al*., 2017; Asledottir *et al*., 2018; Nguyen *et al*., 2019; Lambers *et al*., 2021) not only from milk of A1 variant but also from A2 and other variants of β-casein. Even this BCM 7 peptide was isolated from human milk by Jarmolowska *et al*., (2007). BCM 7 and other peptides have been reported in various types of cheeses (De Noni and Cattaneo, 2010; Sienkiewicz-Szłapka *et al*., 2009). In some studies it has been reported that BCM 7 is responsible for bitter taste of cheese (Shinoda *et al*., 1986). In fermented milk products such as yoghurt, cheese probiotic drinks the amount and sequence of these bioactive peptides depends on the type of microorganisms and processing conditions used for fermentation, maturation and storage. However, the presence of BCM 7 peptide milk and milk products are still unclear.

There are number of processing techniques being used for the manufacturing of dairy products such as heating, fermentation, acid treatment etc. Thermal treatment is widely used processing technique in dairy industry to ensure the microbial safety of milk and milk products. Thermal processing not only ensure microbial safety but also modify physicochemical characteristics of proteins such as maillard browning, denaturation, oxidation, cyclization, racemization etc results in changes in digestibility, bioavailability and release of peptides (Meltretter *et al*., 2007; Pinto *et al*., 2014). Thereby thermal processing may affect the release of BCM 7 in milk and milk products. There are certain studies which reported the presence of BCM 7 in pasteurized, sterilized and UHT milk (Cieślińska *et al*., 2012, Lambers *et al*., 2021). Contradictory to these findings other study reported the absence of BCM 7 in heat processed milk (De Noni and Cattaneo 2010). Miguel *et al*. (2017) reported that thermal treatment did not affect the release of BCM 7 during processing condition. A study reported by De Noni (2008) showed that heat treatment of infant formula did not affect the release of BCM7 after *in-vitro* gastrointestinal digestion. In contrast to this, another study reported that application of heating in liquid infant formula hinders the release of BCM 7 during gastrointestinal digestion (Cattaneo, *et al*., 2017). Very few studies were carried out to evaluate the effect of thermal treatment and release of BCM 7 in milk and milk products and in their gastrointestinal digestive extract (Cieślińska *et al*., 2012; Nguyen *et al*., 2021; Lambers *et al*., 2021).

Another popular processing technique is fermentation in dairy industry, utilized for the preparation of fermented dairy products such as yoghurt, fermented drinks, cheeses, *dahi*. During fermentation various chemical changes takes place such as hydrolysed of proteins to peptides and amino acids by starter culture. A number of bio active peptides generated during fermentation process exhibit a range of bioactivity such as antioxidant, ACE inhibitory, antihypertensive, anticarcinogenic, immunomodulatory, opioid activity etc (Gobbetti *et al*., 2000; Schieber and Bruckner 2000; Donker *et al*., 2007a and 2007b; Otte *et al*., 2011; Kunda *et al*., 2012; Plaisancie *et al*., 2013; Dallas *et al*., 2016; Solieri *et al*., 2018). BCM 7 and BCM 7 like peptides are responsible for opioid activity identified in fermented dairy products. A number of studies reported the presence of pro BCMs peptides such as β-CN (57-68) and β- CN (57-72) (Plaisancie *et al*., 2013; Schieber and Bruckner, 2000); β-CN (58-72), β-CN (59-68), β-CN (59-70) (Kunda *et al*., 2012) and β-CN (57-68), β-CN (57-72), β-CN (59-72) and precursor of BCMs β-CN (57-77), β-CN (57-91) and β-CN (57-93) (Plaisancie *et al*., 2013) in fermented dairy products. Otte *et al*., (2011) also identified β-CN (59-68) and β-CN (59-72) in fermented milk prepared from A2A2 and A1A1 genotype bovine milk by using *Lactobacillus helvetices* respectively. Recently Pro BCMs peptides such as β-CN (58-72), β-CN (59-68) and β-CN (60-68) were isolated in fermented milk prepared by *Lactobacillus casei* and *Lactobacillus rhamnosus* (Solieri *et al*., 2018). Contradictory to these findings some researcher did not observed either pro BCMs or BCM 7 in fermented dairy products (Schieber and Bruckner, 2000; Donker *et al*., 2007a and b, De Noni and Cattaneo, 2010). Yoghurt prepared with *L. delbruekii* ssp*. bulgaricus* SS1 in spite of having number of β-CN derived peptides such as β-CN (6-14), β-CN (7-14), β-CN (73-82), β-CN (74-82) and β-CN (75-82)

(Gobbetti *et al*., 2000) but no BCM 7 like peptide identified by the researchers. Some study reported that BCM 7 is released during gastrointestinal digestion of yoghurt (De Noni and Cattaneo 2010). Where as in recent study conducted by Nguyen *et al*., (2020) did not find any BCM 7 or pro BCMs in yoghurt as well as after gastrointestinal digestion. A number of studies reported the presence of pro BCMs and BCM 7 in varieties of cheeses (Smacchi and Gobbetti 1998; Jarmolowska *et al*., 1999; Jinsma and Yoshikawa 1999, Saito *et al*, 2000; Norris *et al*., 2003; Toelstede and Hofmann 2008; Sienkiewicz-Szłapka *et al*., 2009). De Noni and Cattaneo (2010) identified BCM 7 in Cheddar cheese. Muehlenkamp and Warthesen (1996) and Haileselassie *et al*. (1999) reported that concentration of BCM 7 decreased with storage of cheese. Therefore, BCM 7 released from β-CNs during fermentation may undergo degradation by LAB enzymes during ripening. In addition to LAB culture, mould used for ripening of cheese may influence the proteolytic behavior of the β-CN. The level of BCM 7 in Brie and Rokpol cheese ripened with both mould and LAB were higher compare to bacterial ripened cheeses (De Noni and Cattaneo, 2010; Sienkiewicz-Szłapka *et al*., 2009). However, the role of LAB or mould in generation of BCM 7 is not fully understood. Contradictory to above findings, some studies showed that BCM 7 or other BCMs were not isolated from Brie and Cheddar cheeses (Muehlenkamp and Warthesen 1996; Alli *et al*., 1998). In other study Hayaloglu *et al*., 2010) also mentioned that cheeses made from raw as well as pasteurized milk did not contain BCM 7 or BCMs. Absence of BCM 7 may be its degradation during ripening by microbial amino peptidases (Atlan *et al*., 1990) or may be presence of such peptide at very low concentration. Lactic acid bacteria (LAB) used in the preparation of fermented products posses X-prolyl-di petidyl aminopeptidase (PepX) activity, responsible for the degradation of prolinerich peptides to tri, di or free amino acid residue (Donkor *et al*., 2007 a & b). So probability of presence of BCMs in fermented dairy products is negligible.

Other popular processing technique in dairy industry is acid precipitation for preparation of acid coagulated products such as *paneer*, *chhana*, cottage cheese. Application of acid has significant effect in the milk system and changes physicochemical status of the proteins in acid coagulated dairy products. This may affect the proteolysis and thereby may affect the release of BCM 7 and their identical peptides. Bitri *et al*., (2004) explained that BCM 7 generated by acid treatment in milk. As per our knowledge till date no study has been reported regarding the presence of BCMs in such types of acid coagulated dairy products.

The findings related to the occurrence of BCM 7 in milk and milk products have provided contradictory results. This may be due to differences in the source of milk, processing and storage condition, or possibly due to lack of suitable analytical method. Reversed-phase high performance liquid chromatography (RP-HPLC) and ELISA are most common methods employed for routine detection and quantification of BCM 7 (Jarmolowska *et al*., 2007 and Sienkiewicz-Szłapka *et al*., 2009, Nguyen et al., 2014). RP-HPLC is one of the basic techniques, widely applied in separation of proteins, peptides and amino acids in food products. In RP-HPLC method, the selection of calibration technique plays an important role to get accurate and precise results. Sometimes analyte of interest may lose during sample preparation and extraction (Jessome and Volmer, 2006). Also milk being a complex matrix of heterogeneous molecules, peptides and other micronutrients may interfere in analysis of target analyte. To counter these types of difficulties, preparation of standard solutions in matrix of test sample is best option to counter any matrix effects and loss of target analyte during sample preparation (Nguyen *et al*., 2014). RP-HPLC was used in various studies for quantification BCM 7 in milk and milk products (Muehlenkamp and Warthesen, 1996; Jarmolowska *et al*., 1999; Cieślińska*et al*., 2007). Though, this method has its own limitation as there may be interference from other peptides having similar physical-chemical properties, which can co-elute with target analyte and provide higher values and low sensitivity. Despite its low sensitivity compare to latest techniques, still is a well established, cost effective and easy to operate technique and can be used for routine analysis for identification and quantification of peptides in the matrices of any dairy products.

No systematic studies are available related to processing treatments and BCM 7 formation or degradation. Whether BCM 7 does form at various processing steps or degrades requires further investigation. Therefore in this study, an attempt has been made to standardize RP-HPLC method for quantification of BCM 7 in milk system for routine analysis. This successful validated method has been applied for the identification and quantification of the BCM 7 peptide in milk and milk products and their *in-vitro* digestive extracts to study the effect of different dairy processing condition on generation or degradation of BCM 7. The results obtained by RP-HPLC methods were cross checked by ELISA assay. Simultaneously peptide sequencing analysis was carried out using LCMS for the authentication of BCM 7 in milk products by collecting the representative BCM 7 fraction from RP-HPLC analysis.

CHAPTER –2

Review of Literature

Milk proteins consist of mainly water, proteins, fat, carbohydrates, lactose and minerals. The major protein of bovine milk is caseins and whey proteins, consisting nearly 80% and 20% of total protein, respectively. Casein is the insoluble part and composed of four fractions namely: α_{s1}, α_{s2}, β and κ-CN, exists in form of micelles. The α_{s1}, α_{s2} and β-CN fractions are located in the core region of the micelle and κ-CN present on the micelle surface (Considine *et al.*, 2007). The caseins fraction is originally considered as merely a macronutrient source, but during recent decades, a number of studies have indicated that there may be considerable impact on health from casein derived bioactive peptides. Bioactive peptides are encrypted within the protein sequence in raw milk. They are generally released during processing and enzymatic action, and exert an impact on cells or tissues of the body. Bioactive peptides derived from caseins have been associated with a range of different health-related effects, and several reviews have been published on this matter (Thiruvengadam *et al*., 2021; Clare and Swaisgood, 2000 and Silva and Malcata, 2005).

2.1 Casein Polymorphism

The bovine casein is highly polymorphic in nature. Casein fractions of milk proteins posses various genetic variants (Farrell *et al*., 2004 and Kaminski *et al*., 2007) such as

1- α_{s1}-Casein: A, B, C, D, E, F, G, H

2- α_{s2}-Casein: A, B, C, D

3- β- Casein: A1, A2, A3, B, C, D, E, F, G, H1, H2, I, H

4- κ-Kappa Casein: A, B, C, E, F1, F2, G1, G2, H, I, J

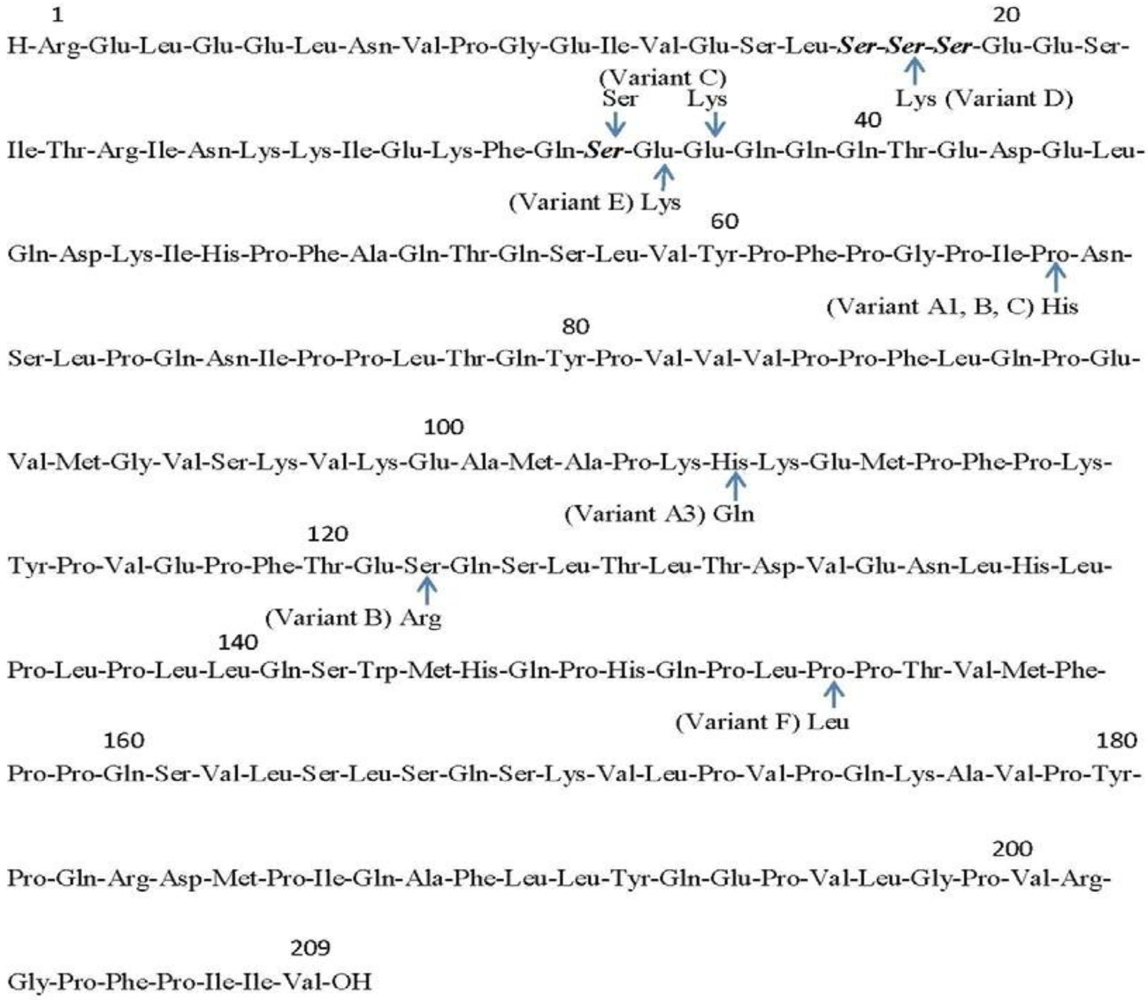


Fig. 2.1. Primary structure of β-CN A2 variant indicating amino acid substitutions of the other variants (Swaisgood 2003)

2.1.1. β-CN and A1 and A2 Milk

β-CN posses 209 amino acids residues in peptide sequence. It is a highly polymorphic fraction of casein and exists in 15 variants in bovine milk (Fig. 2.1), namely β-CN A1, A2, A3, A4, B, C, D, E, F, H1, H2, I and H (Kaminski *et al*., 2007, Gallinat *et al*., 2013). β-CN A1 and β-CN A2 are most common variant form of β-CN. A1 and A2 variants are differ with each other in a single amino acid at position '67'. β-CN A2 consist of proline at 67 position where as β-CN A1 variantcontains histidine at same position.

Originally, β-CN A2 is the native variant of β-CN in first domesticated bovine (*Bos* genus) (Daniel *et al.*, 1998). A1 β-CN variant commonly found in bovine milk of western world, especially in European and American breed.

These bovine breed belongs to the Taurine cattle named as *Bos Taurus*. Whereas β-CN A2 variant found in milk from Asian bovine belongs to Zebu cattle known as *Bos indicus* (Mishra *et al*., 2009). It is believed that due to some point mutation in β-CN A2 about 5000 years ago (Fig. 2.2) might be responsible for the generation of β-CN A1 variant in European Taurine cattle probably somewhere near to Anatolia (Loftus *et al*., 1999 and Troy *et al*., 2001).

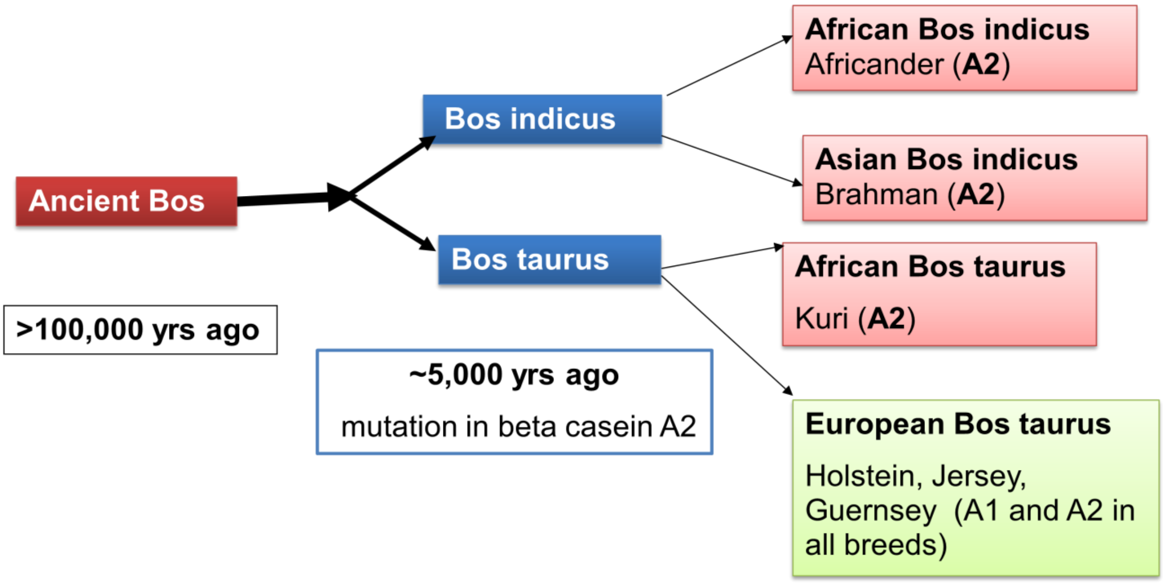


Fig. 2.2. Breed evaluation and β-CN type (Fox and McSweeney 2003 and Daniel *et al.*, 1998)

The Indian breed such as Red Sindhi, Sahiwal, Tharparkar, Gir and Rathi posses 100 % A2 allele gene and hence produces only β-CN A2 casein milk. The other Indian cattle breed contains about 94% A2 allele gene (Joshi, 2011), where as foreign breed such as Friesian (HF), Jersey posses around 60% A2 allele gene (Ganguly *et al*., 2013). The crossbred cattle such as Karan Fries (Tharparkar X Holstein-Friesian) and Karan Swiss consist of 79-89% of A2 allele gene frequency (Ul Haq *et al*., 2012). Holstein-Friesian cows produce milk containing predominantly β-CN A1 variant (Tailford *et al*., 2003), while Guernsey and Jersey breeds produce more β-CN A2 than β-CN A1 variant (Bell *et al*., 2006). The both β-CN variants are very closely similar to each other and have sequence homology at residues 60-66. The single amino acid difference at position 67 makes a significant change in properties of β-CN, as bonding between histidine and its adjacent amino acid in β-CN. A1 variant is much weaker and can be easily hydrolyzed between the amino acids residues 66-67 by proteases and processing condition and break down to peptide of seven amino acids residues known as BCM 7, whereas β- CN A2 variant is resistant to hydrolysis at these residues due to stronger bond association between proline and adjacent amino acid (Thiri *et al*., 2012; Jinsmaa and Yoshikawa, 1999).

2.2 Beta-casomorphins and their release

Beta-casomorphins (BCMs) are a group of peptides released after successive gastrointestinal enzymatic hydrolysis of bovine β-CN (Jinsmaa and Yoshikawa, 1999; De Noni and Cattaneo, 2010 and Boutrou *et al*., 2013) at amino acid positions 60- 70 (Fig. 2.3). They are opioid in nature and posses morphine like activity. These peptides consist of a sequence of 4-11 amino acid residues (Kaminski *et al*., 2007). The first three amino acid residues are similar in all peptides and consist of -Tyr-Pro-Phe- (Muehlenkamp and Warthesen, 1996). Among these peptides BCM 7 is most extensively studied peptides with the highest opioid activity (Brantl *et al*., 1981, Kálmán *et al.,* 1992 and Gobbetti *et al*., 2002). BCM 7 consists of seven amino acid residues Tyr- Pro-Phe-Pro-Gly-Pro-Ile (YPFPGPI). The amino terminal of BCM 7 (Tyr-Pro-Phe) acts as opioid (Meisel and Fitz-Gerald, 2000). The rest four amino acid residues have sequence homology with beta cell specific glucose transporter GLUT-2 molecule and may possibly responsible for type I diabetic condition (Woodford, 2009).

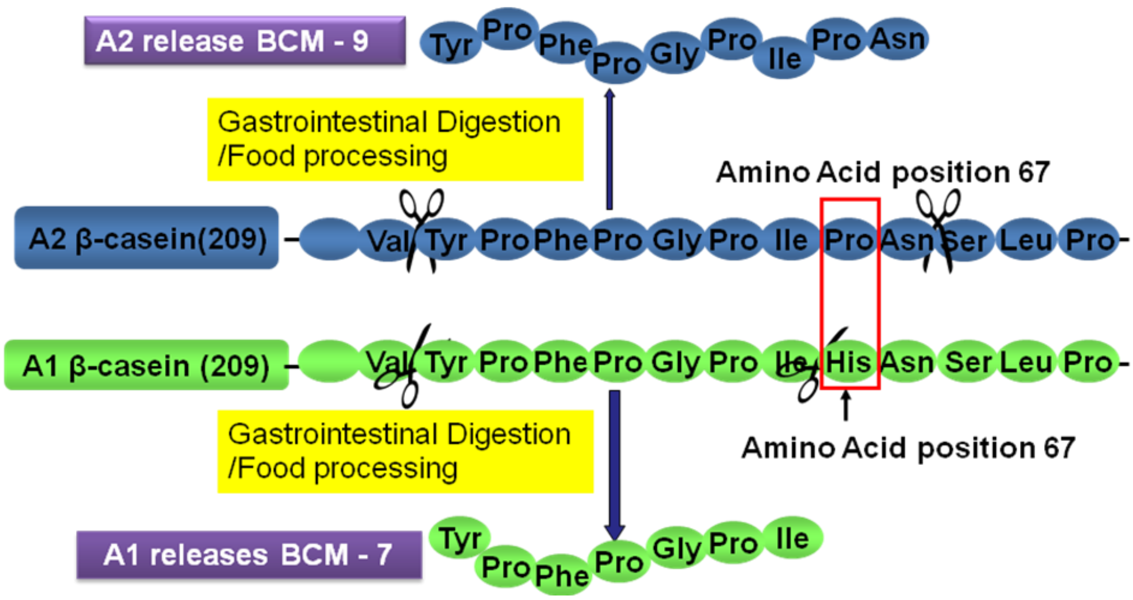


Fig.2.3. Mechanism for release of BCM 7 peptide (Roginski 2003 and Kostyra *et al.,* 2004)

A number of studies have described that BCM 7 is releases from β- CN A1 variant during *in- vitro* and *in-vivo* digestion (De Noni 2008, Boutrou *et al*., 2013, Barnett *et al*., 2014, Ul haq *et al*., 2015). BCM 7 is cleaved from β-CN at amino acid positions 60- 66 (Fig. 2.3) during *in-vitro* enzymatic hydrolysis by pepsin, pancreatic elastase and leucine amino peptidase. BCM 7 is hydrolysed only from β-CN A1 variant by pepsin *in- vitro* (De Noni 2008 and Jinsmaa and Yoshikawa, 1999) (Fig. 2.4).

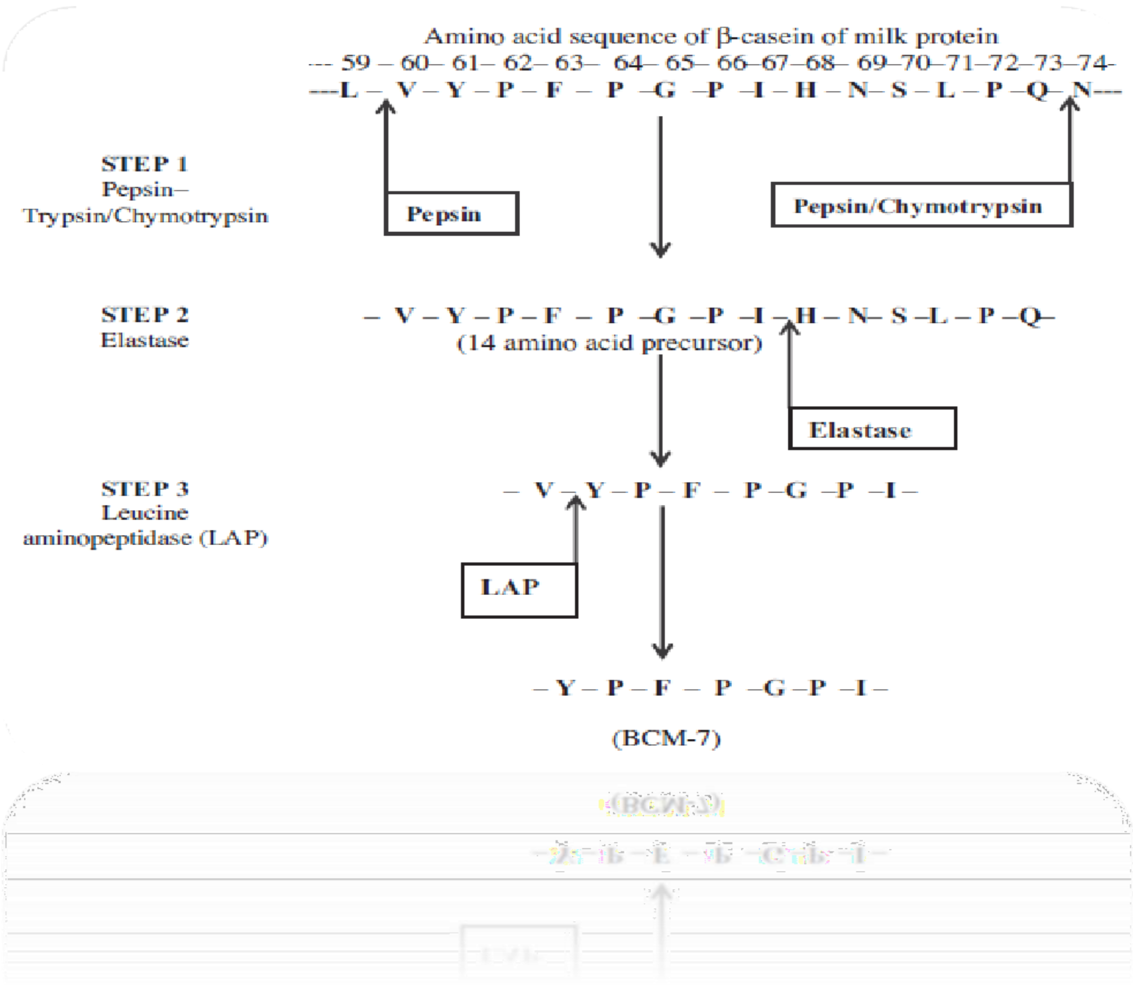


Fig. 2.4. Step wise release of BCM 7 during enzymatic hydrolysis (Jinsmaa and Yoskikawa 1999)

Elastase cleaves peptide bond between isoleucine (Ile) and histidine (His) and releases carboxyl terminal BCM 7 peptide. Whereas Pepsin and leucine amino peptidase is require to release theamino terminal of peptide. In some study it has been demonstrated that BCMs also releases on mild acid hydrolysis at pH 2.5, 37^{0}C with pepsin and trypsin. Pepsin hydrolysis of unprocessed milk followed by digestion with corolase also releases BCM7 (Bitri, 2004, Hernandez-Ledesma *et al*., 2007). Cieślińska *et al*., (2012) demonstrated that during simulated gastro intestinal digestion of beta casein A1 variant using cocktail of enzymes (pepsin, trypsin and elastase) at acidic pH releases BCM 7. Elastase is the main enzyme responsible for release of this peptide. During ex-vivo digestion of beta casein variants by gastric juice, about 109 peptides are released. Pepsin hydrolyzes the proteins preferably at Phe, Lys and Tyr amino acid position. While duodenal hydrolysis cleaved the peptide at Thr, Gln, Leu, Val and Pro site and generates BCM 7. The extent of BCM 7 release is depends on the β-CN variants; it is formed quickly in A1 variant (5 min) compare to A2 variant (120 min) (Asledottir *et al*., 2017). It has been reported that generated BCM 7 is further hydrolyzed by brush border peptidase (dipeptide peptidase- 4) before reaching the target organ (Osborne *et al*., 2014).

2.2.1. BCM 7 and health implications

A number of studies have reported that milk polymorphisms may also affect human health beside their effect on milk traits. There are strong correlations exist between consumption of high β-CN A1 variant containing milk and type 1diabetes and ischemic heart diseases (Elliott *et al*., 1999; McLachlan, 2001). The main cause of health problems related with this variant is due to formation of BCM 7 during digestion. BCM 7 is an opioid in nature and exerts morphine like effect similar to narcotics. Consumption of β-CN A1 diet related to the pro-inflammatory action especially in colon due to opioid effect of released BCM 7 (Barnett *et al*., 2014). In another study it is reported that consumption of β-CN A1 diet is capable to induce inflammatory response in gut by activating Th2 pathway compared to β-CN A2 diet (Ul Haq *et al*., 2014). BCM 7 is also act as an oxidant and causes damage to low density lipoproteins (Laugesen and Elliott 2003 and Tailford *et al*., 2003). Moreover consumption of A1 milk has also been linkedto the neurological symptoms such as autism and schizophrenia in animals and human (Sun and Cade 1999 and Cade *et al*., 2000, Thiruvengadam *et al*., 2021). In another study it is observed that the autistic children have significantly higher concentration of bovine BCM 7 in their urine sample than their control group and severity of the symptoms are in correlation with the BCM 7 concentration (Sokolov *et al*., 2014). BCM 7 also causes sudden infant death syndrome in new born infants (Sun *et al*., 2003). BCM 7 is quite resistant to further breakdown and cannot absorb in body as intestinal wall are impermeable to BCM 7. In certain cases such as person suffering from celiac disease or in infant which has higher intestinal permeability, BCM 7 can easily permeate through the intestinal membrane and causes certain adverse effect in body (Cade *et al*., 2000). Recent study demonstrated that β-CN A1 variant is also related to milk intolerance condition. Diet with β-CN A1 variant worsens the gastrointestinal disorder, increase GI transit time and increases serum inflammation condition along with lactose intolerance. Whereas consuming A2 milk diet did not posses such types of effects (Jianqin *et al*., 2016). However European Food Safety Authority (2009) published a report and stated that studies performed to date did not allow a conclusion on the role of BCM 7 in development of type-1 diabetes nor A1 beta casein as a causative factor for various types of health problems.

2.3 Effect of different processing condition on β-casomorphins and other peptides

2.3.1 Thermal processing

Heat treatment is an important processing technique in dairy industries to get safe and quality food. Heat treatment destroys pathogenic and spoilage microorganism also causes change in physiochemical properties of milk, especially due to denaturation of whey proteins. When milk is heated above 70°C there is a significant denaturation of two major whey protein, β-lactoglobulin and α- lactalbumin (Considine *et al*., 2007). The denatured β-lactoglobulin gets attached to casein micelles, particularly the κ-CN on the micelle surface of casein. This forms a complex of κ-CN~β-LG, which affects the physicochemical characteristics of milk and milk products (Krasaekoopt, 2003). Apart from that, thermal treatment also induces protein fragmentation and form lower molecular weight peptides (Gaucheron *et al*., 1999). Meltretter *et al*. (2008) reported that on heating of milk, there is formation of five new peptides from casein. Generated peptides of unknown structure have mass-to-charge (*m/z*) ratios of 974.4, 2218.7, 3730.1, 4297.8 and 4436.8, but none of peptide is BCM 7. Contradictory to this study Cieślińska *et al*., (2012) reported that heating of milkat 90^{0}C for 20 min (pasteurization) causes significant increase of BCM 7 compare to raw milk. During sterilization (117 °C/ 5 min), the BCM 7 content increased about 2 to 3 fold. The formation of BCM 7 or other peptides may be due to thermal treatment, enzymes or acid hydrolysis. But author justified that probability of hydrolysis of proteins by enzymatic action or acid during thermal processing of milk is rare and can be excluded because enzymes gets inactivated at higher temperature and the change in pH of heated milk is nearly insignificant compared to the raw milk. However, heat treatment causes non enzymatic browning i. e. Maillard reactions that may form free radicals which may attacks the peptide bonds and cleave the bonds resulting in the release of peptides (Meltretter *et al*., 2008). The strength of peptide bonds is an important aspect for fragmentation of protein by thermal treatment. Gaucheron *et al*. (1999) have reported that heating of raw milk at 120^{0}C for 30 min librated about ten peptides. Out of ten peptides two peptides are generated from β-CNs, including β-CNf1-14 and β-CNf1-16, but neither BCM 7 nor pro-BCMs were reported. The peptides were generated during thermal treatment is due to cleavage of bonds at aspartic acid (Asp), asparagine (Asn) or glutamic acid (Glu). Thus peptide bonds involving these amino acids are more susceptible to cleavage by thermal treatment (Hustinx *et al*., 1997). Gaucher *et al*., (2008) identified a number of pro-BCMs in UHT milk. The identified fragments are β-CN f (54-68), β-CN f (54-69), β- CN f (55-65), β-CN f (55-68) and β- CN f (57-68), and recommended that these peptides generated from the action of elastase and cathepsin enzymes. The three peptides i. e. β-CN f (54-68), β-CN f (55-68) and β-CN f (57-68) generated due to cleavage of peptide bonds involving the Asn residue at position 68. Therefore, this finding may suggest that peptide bonds containing Asn are more prone to enzymatic hydrolysis if milk is heated. A number of studies have showed that the fragment β-CN f (57-68) and β-CN f (59-68) both contain Asn residue at position 68, are also reported in yoghurt (Schieber and Bruckner, 2000; Kunda *et al*., 2012 and Plaisancie *et al*., 2013). The formation of these two peptides takes place during heat treatment of milk rather than fermentation and storage stages. Benfeldt and Sorensen (2001) and Mendia *et al*., (2000) also reported that pasteurization of milk causes increase in proteolysis of β-CNs in the cheese prepared from this milk. This phenomenon may be linked to heat-induced activation of plasmin, one of the native enzymes in milk which is quite heat stable. In principle, when milk is pasteurised, activity of plasmin increases due to thermal inactivation of plasminogen inhibitors. Hence plasmin acts on β-CN (Sousa *et al*., 2001) and generated large number of peptide fractions. The primary cleavage sites of plasmin on beta-casein are Lys_{28}-$Lys2_{9}$, Lys_{105}-His_{106}

and Lys_{107}-Glu_{108}, cleavage of which yields f (29–209), f (106–209), f (108–209), f (1–105), f (1–107), f (1–28), f (29–105) and f (29–107). Not only plasmin other still active proteases also acts on β-CN and causes extensive hydrolysis. In contrast to above studies Hayaloglu *et al*., (2010) reported that pasteurization (72 °C/ 30 s) of milk before preparation of cheese resulted in the decrease in peptide content compared to the cheese made from raw milk; however, generated peptide are not reported in the study. The heat treatment of milk at 72 °C / 30 s destroys the non-starter micro flora resulting in lesser number of peptides in cheese prepared from pasteurised milk compared to cheese made from raw milk. Similarly Miguel *et al*. (2017) reported that thermal treatment did not affect the release of BCM 7 during processing condition. In contrast to this, another study reportedthat application of heating in liquid infant formula hinders the release of BCM 7 during gastrointestinal digestion (Cattaneo, *et al*., 2017).

Very few studies were carried out to evaluate the effect of thermal treatment and release of BCM 7 in milk and milk products and in their gastrointestinal digestive extract. Recently Lambers *et al*. (2021) studied the processing effect on release of BCM 7 during different digestion stages and reportedthat no pro BCMs and BCM 7 were released in raw and heat processed milk during gastric digestion. Similar findings were also reported by Asledottir *et al*. (2017) and De Noni (2008).

2.3.2. Fermentation and storage

Fermentation is an important processing technique in the production of fermented milk products such as dahi, cultured milk, yoghurt, cheese etc. During fermentation milk sugar is converted into lactic acid and milk fat into fatty acids, and proteins hydrolysed to peptides and amino acids by starter microorganism. For fermentation variety of microorganism are utilized such as bacteria, mould and yeast. The extent and kinetics of these reactions depends on the type of microorganism used for fermentation, temperature, time of incubation, pH condition and time of storage. The criteria for selecting the strains of culture play a vital role in determining the characteristics of resulted products including the formation of different peptides in the final products (Jarmolowska, 2012). Yoghurt prepared with *L. delbruekii* ssp*. bulgaricus* SS1 contains different types of β-CN derived peptides such as β-CN f (6-14), β-CN f (7-14), β-CN f (73-82), β-CN f

(74-82) and β-CN f (75-82) (Gobbetti *et al*., 2000). Papadimitriou *et al*. (2007) also identified the some peptides such as β-CN f (176-180) and β-CN f (1-8) in yoghurt prepared with same culture. *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* are most common stain used for the production of yoghurt and have been characterized in commercial yoghurt (Schieber and Bruckner, 2000; De Noni and Cattaneo, 2010; Jarmolowska, 2012). The yoghurt sample stored for 21 and 30 days at low temperature did not contain BCM 7. Hence this finding showed that culture *L. delbrueckii* ssp*. bulgaricus* and *S. thermophilus* is not able to generate BCMs even on storage up to 1 month. Same findings were reported by De Noni and Cattaneo (2010) that 30-35 days stored cheese prepared from *L. delbrueckii* ssp*. bulgaricus* and *S. thermophilus* did not contain BCM 7. Recently Pro BCMs peptides such as β-CN (58-72), β-CN (59-68) and β-CN (60-68) were identified in fermented milk prepared by *Lactobacillus casei* and *Lactobacillus rhamnosus* (Solieri *et al*., 2018). In contrast to these studies Donkor *et al*., (2007) had reported a number of β-CNs derived peptides in probiotic yoghurt prepared with *L. delbrueckii* ssp*. bulgaricus* and *S. thermophilus*, and probiotic bacteria *L. casei, L. acidophilus* and *Bifidobacterium lactis*, but BCM 7 is not identified. Where as in recent study conducted by Nguyen *et al*., (2020) did not report any BCM 7 or pro BCMs in yoghurt and in their gastrointestinal digestive extracts. Jarmolowska (2012) identified BCM 7 in commercial traditional and probiotic yoghurt sample and reported that concentration of BCM 7 is decreases with storage. The hypothesis behind this is that BCM 7 is a proline rich peptide and highly susceptible to LAB derived PepX that preferentially cleaves peptide bonds involving proline. Atlan *et al*., (1990) reported that PepX is present in the cytoplasm and releases into the surrounding medium on destruction of bacteria cells. At low pH and low temperature storage condition of yoghurt, bacterial cells are more prone to lysis and release more PepX (Otte *et al*., 2011). Hence decrease in BCM 7 during storage of yoghurt is reported. Whereas during fermentation, proteases present on the cell wall of bacteria, hydrolysed the caseins into larger peptides, and further hydrolysed to di and tri-peptides in-side the bacterial cell (Nielsen *et al*., 2009). Consequently, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* may cleave β-CNs into BCM 7 and other related BCMs during fermentation and subsequently degrade them into even smaller peptides during storage. However study conducted by Paul and Somkuti (2009) demonstrated that, fermentation with *L. delbrueckii* ssp. *bulgaricus* causes significant hydrolyses of antimicrobial and hypertensive peptides after 4 hours incubation at pH 4.5 condition. This study suggest that any BCMs generated during fermentation or pre-existing in the raw milk before yoghurt making may be degraded during cold storage at pH 4.5.

2.4 Presence of beta-casomorphins in milk and milk products

2.4.1 Raw milk

Bovine milk often contains somatic cells, native milk enzymes or bacteria contaminated from external environment. These are three factors that can contribute to the hydrolysis of proteins to peptides. Somatic cell count (SSC) in milk is an important factor for assessment of the severity of mastitis which indicates the inflammation of cow udders. Milk is considered as coming from a mastitis infected cow if somatic cells exceed 500,000 per milliliter (Ginn *et al*.,1985). Many studies have measured BCM 7 in milk collected from cows with different severities of mastitis. This peptide is found in fresh cow milk, in which somatic cells are not characterized (Cieślińska *et al*., 2007). Similarly, Cieślińska *et al*., (2012) identified BCM 7 in cow milk collected from healthy cows that does not indicate any sub-clinical symptoms of mastitis. In contrast, Napoli *et al*., (2007) have reported that BCM 7 is undetectable at any level in milk collected from cows with mastitis. Consequently, somatic cells are nota factor associating with the presence of BCM 7 in fresh milk. Endogenous proteases often present in milk during lactation consist of plasmin, cathepsin and elastase. According to Weinstein and Doolittle (1972), plasmin specifically cleaves the peptide bonds between lysine and arginine residue. There is no lysine or arginine amino acid residue at positions from 59-70 on β-CN chain. In addition, plasmin hydrolyses peptide bonds on β-CN at amino acid residues Lys_{28} and Lys_{29}, Lys_{105} and His_{106}, and Lys_{107} and Glu_{108} (Aslam and Hurley, 1998). Meanwhile, cathepsins and elastase breaks down β-CNs to pro-beta casomorphins (pro-BCMs) such as β-CN f (54-68), β-CN f (55-68), β-CN f (57-68) and β-CN f (55-65), but no BCMs (Gaucher *et al*., 2008). More interestingly, cathepsin B cleaves β-CNs to BCM 10 and an undefined peptide (β-CN f 60-) (Considine *et al.*, 2004). Investigations of the occurrence of BCM 7 and other BCMs in human milk may also assist in our understanding of the origin of formation of these peptides in bovine milk. Jarmołowska *et al*., (2007) identified BCM 5 and BCM 7 in human milk that is collected from healthy women and stored at -70 °C immediately after expression. Obviously, in these conditions of collection and storage of milk samples, the hydrolysis of proteins by somatic cell or bacteria- derived enzymes is very unlikely. Therefore, BCM 5 and BCM 7 are probably released from β-CNs in the mammary glands by endogenous proteinases before milk is expressed (Koch *et al*., 1988). However, the identity of specific endogenous enzymes in human milk and their mechanism of action are unknown. The presence of β-CN variants and their ratio in milk plays an important role in the formation of BCM 7 and other BCMs. As previously described, *in- vitro* studies demonstrated that BCM 7 is only released from β- CN A1 variant, but other BCMs can be released from both β-CN A1 and β-CN A2 variant (Jinsmaa and Yoshikawa, 1999). Surprisingly however, BCM 7 has been found in milk containing not only pure β-CN A1 variant, but also β-CN A2 and β-CN A1/A2 (Cieślińska *et al*., 2012). The level of BCM 7 is the highest in milk containing only β-CN A1 variant, followed by in milk containing β-CN A1/A2 variant and then β-CN A2 variant. In this regard, BCM 7 may be formed from β-CN A2 variant at very low rate (Korhonen and Marnila, 2013). Recently some studies reported the occurrence of BCM 7 not only in A1A1 variants of milk but also in A2A2, and other variants of β-CN (Asledottir *et al*., 2017, Miguel *et al*., 2017; Asledottir *et al*., 2018; Nguyen *et al*., 2019; Lambers *et al*., 2021). Similarly Nguyen *et al*. (2019) identified BCM 7 in raw milk of Holstein Friesian breed. This bovine breed majorly contains A1A1 type of β-CN variant.

2.4.2. Heat processed milk

Heat-treated liquid milk is a product wildly consumed across the world. It consists of pasteurised milk (75^0C/15s), UHT milk (UHT-149^0C/2s) and sterilized milk (110^0C/10min). In addition to destroying bacteria, heat treatment of milk also changes its physico-chemical properties; for instance, increasing viscosity, browning of colour, changes in protein conformation and release of peptides. BCM 7 has been found in commercial UHT milk (Juan-Garcia *et al*., 2009 and Thiri *et al*., 2012) and in pasteurised and sterilized milk (Cieślińska *et al*., 2012). In contrast, De Noni and Cattaneo (2010) did not identify BCM 7 in similar milk products. Another study reported the five new peptides identified in cow milk when milk is heated at different time temperature combination i. e. 72, 85 and 120 °C for 10, 20 and 30 min respectively, but contain neither BCM 7 nor other BCMs (Meltretter *et al*., 2008). Whereas some other researchers reported that BCMs were not found in commercial UHT and pasteurised milk (Gaucher *et al*., 2008 and Meltretter *et al*., 2008). Recently Miguel *et al*. (2017) reported that thermal treatment did not affect the release of BCM 7 during processing condition. In contrast to this, another study reported that application of heating in liquid infant formula hinders the release of BCM 7 during gastrointestinal digestion (Cattaneo, *et al*., 2017). Recently Lambers *et al*. (2021) studied the processing effect on release of BCM 7 during different digestion stages and reported that no pro BCMs and BCM 7 were released in raw and heat processed milk during gastric digestion. Similar findings were reported by Asledottir *et al*. (2017) and De Noni (2008).

2.4.3. Yoghurt

Yoghurt is a dairy product mainly produced from bovine milk by fermentation using lactic acid bacteria (LAB) *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* and other probiotic culture. Recently, BCM 7 has been investigated in commercial yoghurt or fermented milk. De Noni and Cattaneo (2010) reported that yoghurt and other fermented milk products do not contain BCM 7. In contrast, Jarmolowska (2012) reported the presence of BCM 7 in traditional and probiotic yoghurt. A number of different peptides are also formed from β-CNs during traditional and probiotic yoghurt production (Donkor *et al*., 2007; Kunda *et al*., 2012). Various identified peptides are pro-BCMs such as β-CN f (57-68) and β-CN f (57-72) (Plaisancie *et al*., 2013; Schieber and Bruckner, 2000); β-CN f (58-72), β-CN f (59-68), β-CN f (59-70) (Kunda *et al*., 2012) and β-CN f (59-72) (Plaisancie *et al*., 2013). In principle, these pro-BCMs may be further degraded by peptidase system of yoghurt starter cultures into BCMs during storage. According to Kunda *et al*. (2012) two di- peptides, probably β-CN f (60-61) and β-CN f (62-63), cleaved from both β-CN A1 and β-CN A2 variants are identified in commercial yoghurt. Residues at position 61 and 63 on the chain of parent β-CNs are proline residues. The peptide bonds between proline and other amino acids are hydrolysed by the enzyme PepX that is released from Lactic acid bacteria (LAB) (Gobbetti *et al*., 2002). Therefore, the finding of Kunda *et al*. (2012) indicates that BCM 7 and other BCMs are likely to be hydrolysed into smaller peptide fragments during yoghurt fermentation or storage. Therefore, in yoghurt fermentation there may formation of BCM 7 and other BCMs by *L. delbrueckii* ssp. *bulgaricus* with their subsequent degradation by *S. thermophilus*. It is unknown that when BCMs ar releases or degrades in yoghurt processing by the starter cultures. Therefore, this interaction needs further investigation.

2.4.4 Cheese

Cheese is a dairy product produced by coagulation of casein protein in milk. During cheese production, milk is heated, acidified by inoculation with starter culture and the addition of the enzyme rennet to coagulate caseins to give curd and whey. Further curd is separated out from whey and pressing is done to release the residual whey followed by ripening of the curd into the final cheese. Lactic acid bacteria are often used as a starter culture for fermentation of milk during cheese making. In the manufacture of Cheddar, starter cultures that are commonly used for fermentation include a mixture of *Lactococcus lactis* ssp*. cremoris* and *Lactococcus lactis* ssp*. lactis* (Robinson, 1995)*.* In addition to producing lactic acid to coagulate milk protein, a number of biochemical changes takes place during cheese making. Proteolysis is complex biochemical reaction that occurred duringripening process involving the formation of rather large well-defined peptides, and their subsequent hydrolysis into smaller peptides and free amino acids. These changes in protein molecules have significant role in the development of characteristics flavour in Cheddar cheese. During ripening, proteolysis in cheese is carried out by rennet, milk indigenous milk proteinases, enzymes from the starter, and nonstarter, or secondary cultures (Sousa, Ardo, McSweeney, 2001) The extent and pattern of proteolysis dependupon the quality of rennin, strains of LAB, indigenous milk enzymes and environmental conditions during cheese ripening. Primary proteolysis initially carried out by rennet enzyme (chymosin) and up to some extent by plasmin by cleaving peptides bonds in β-CN involving basic and aromatic amino acid in similar manner as of plasmin, elastase and cathepsin and released large molecular weight peptides. Large peptides are further hydrolyzed by starter microbial culture and non-starter microorganism of the cheese known as secondary proteolysis to small chain peptides (Fig. 2.5). A number of bioactive peptides released in cheese during fermentation and ripening process. The formation or degradation of BCM 7 and other BCMs may depend on the strains of LAB culture and cheese making conditions. In addition, using different starter cultures for fermentation or moulds for ripening also contributes to variations in the peptide profile in the ripened cheese.

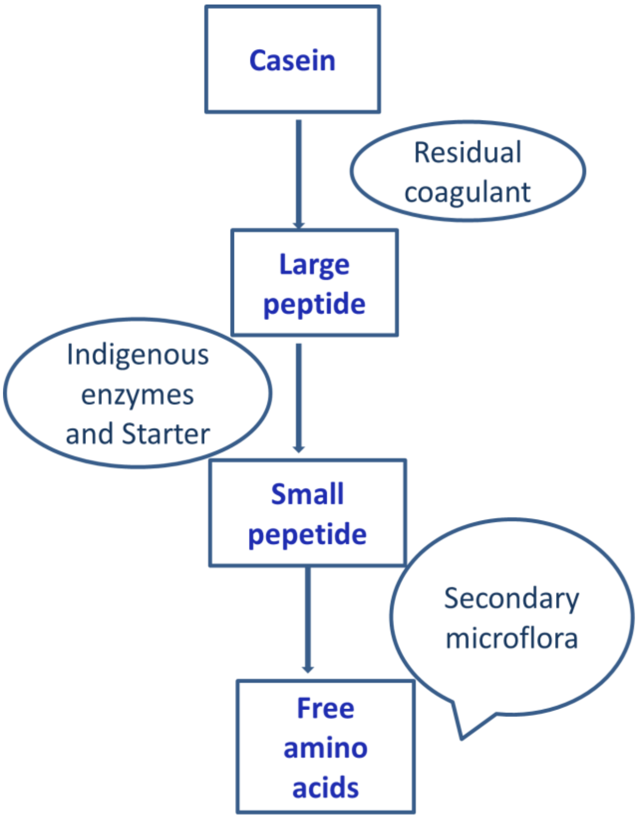


Fig. 2.5. Proteolytic mechanism during preparation of cheese

In many varieties of cheese, specific moulds are also added during ripening. Milk usedfor cheese making is commonly pasteurised at 72^{0}C for 15s (Hayaloglu *et al*. 2010).

However, some varieties of cheeses are produced from milk without heat treatment or higher temperature heating than pasteurisation (>72^{0}C). Heat treatment of milk causes denaturation of the whey proteins, beta-lactoglobulin (β-LG) and alpha-lactalbumin (α-LA), leading to interaction between caseins and denatured whey proteins. This interaction not only leads to increase in the yield of cheese, but also significantly affects hydrolysis of proteins during ripening, impacting the flavour and texture of final products (Benfeldt and Sorensen, 2001). In general, protease systems cleave proteins into a number of larger peptides during ripening. Figure 2.6 shows the enzymatic cleavage pattern of β-CN in cheese manufacturing process. Milk enzyme as well as microbial enzymes acts on β- CN and subsequently hydrolysed the whole peptide into smaller peptides even free amino acids (Benfeldt and Sorensen, 2001). A large number of pro BCMs are generated during cheese processing, which may further hydrolysed to tetra, tri or di- peptide by enzyme system of lactic acid bacteria. A number of studies reported the presence of pro-BCMs and BCM 7 in varieties of cheese.

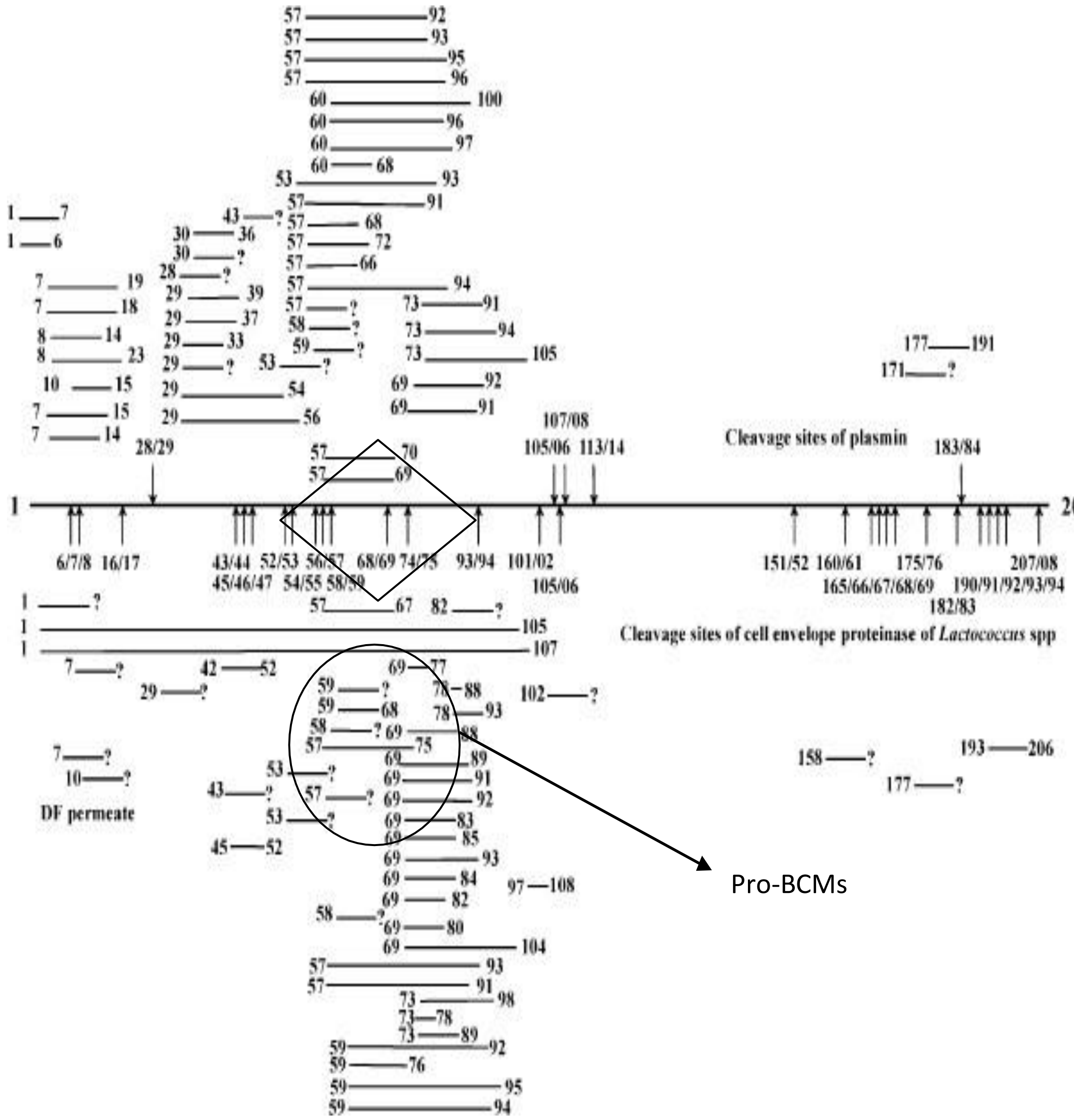


Fig 2.6. Cleavage pattern of β- CN in cheese (Sousa *et al.,* 2001)

The peptide β-CN f (58-72) called pro-beta casomorphin (pro-BCM) is reported in Crescenza cheese after 8-10 day ripening (Smacchi and Gobbetti, 1998), Cheddar and Jarlsberg cheese (Stepaniak *et al.*, 1995). This peptide may be cleavage by peptidases into beta-casomorphin 13 (BCM 13), β-CN f (60-72) as reported by Jinsmaa andYoshikawa (1999), or into other smaller BCMs if the cheese is ripened for a longer period. In recent years, the presence of BCMs in cheese, especially BCM 7, has been investigated (Nguyen *et al.,* 2014; De Noni and Cattaneo 2010; Sienkiewicz-Szłapka *et al*., 2009). In contrast, some studies shows that BCM 7 or other BCMs are not found in Brie and Cheddar cheeses (Alli *et al*., 1998). These findings may indicate that BCM 7 is not probably formed from β-CNs, or may be further degraded during cheese ripening or might be present at a low level that cannot detect by analytical method used for quantification. According to McSweeney (2004), rennet enzymes (chymosin) also cleaved β-CNs in a similar manner to plasmin, elastase and cathepsin. Therefore, any single endogenous protease or chymosin is incapable of releasing BCM 7 or other BCMs during cheese ripening. However, a combination of these enzymes and others enzymes derived from lactic acid bacteria or mould may play a role in formation of BCMs and the BCM-releasing activities; however, this needs further investigation. The BCM 7 and other related BCMs in cheese could not originate from the milk, because if so, these peptides would be removed in the whey fraction during washing of the curd (Jarmolowska *et al*., 1999). De Noni and Cattaneo (2010) reported the BCM 7 at 0.11 ng/kg level in Cheddar cheese. Muehlenkamp and Warthesen (1996) showes that BCM 7 level decreases by 50 % after 6-15 weeks of storage when it is incubated with *Lactococcus lactis* ssp. *cremoris* at pH 5.0 and 1.5 % NaCl. These conditions of incubation and storage are similar to those used in production of Cheddar cheese. According to Haileselassie *et al.* (1999) BCM 7 is detected in emulsified Cheddar cheese with added Neutrase, an enzyme produced from *Bacillus subtilis*, after eight hour incubation. However, the produced BCM 7 is degraded by peptidases from *L. casei, Lactococcus lactis* and *Aspergillus oryzae* after 72 h incubation. Therefore, BCM 7 may be generated from hydrolysis of β-CNs during fermentation, followed by degradation into smaller peptides by

LAB during ripening. In addition to starter culture, mould is also used for ripening in some cheeses such as Brie, Camembert or Gorgonzola. This microorganism strongly influences the appearance, flavour and texture of these cheeses (Wilhelm, 1999). According to Takafuji (1993), mould-derived proteases play a role in degradation of β-CNs into large peptides, once the level of activity the enzymes increased after 20 day ripening. Subsequently, large peptides are hydrolysed into smaller ones by LAB derived enzymes (Fig. 2.7).

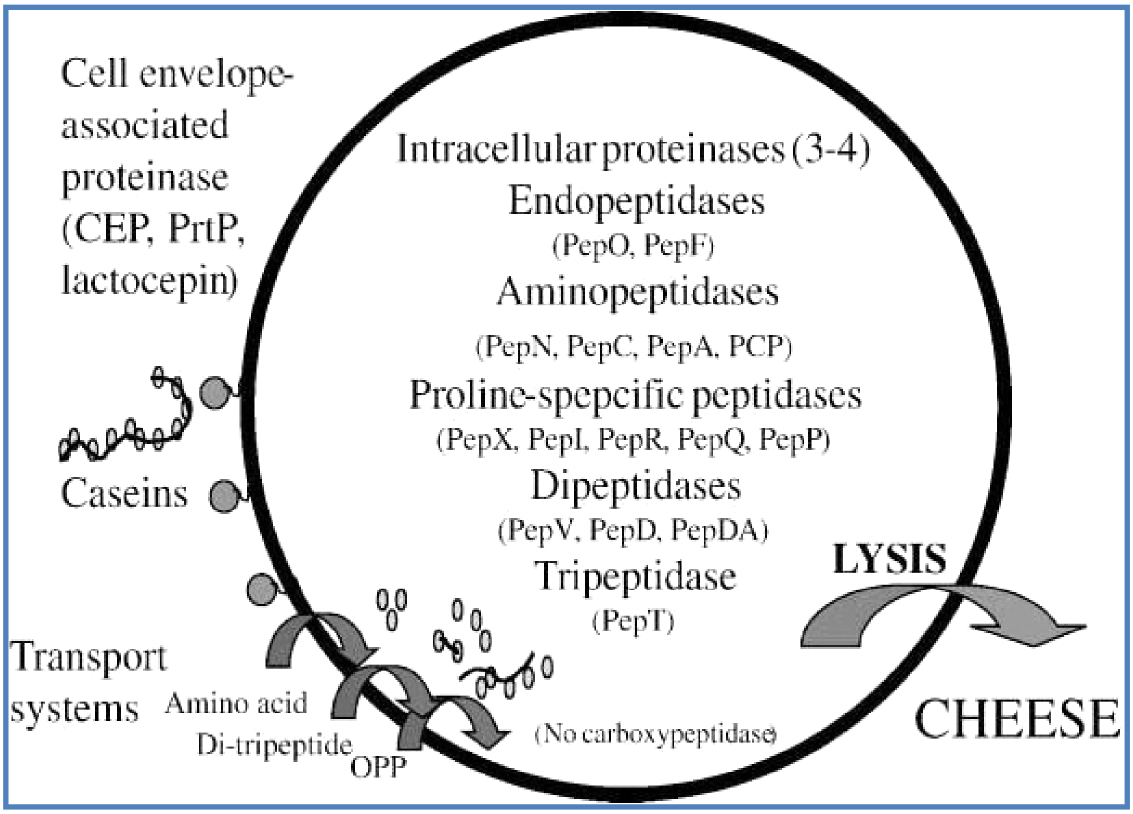


Fig. 2.7. Role of lactic acid bacterial enzymes in further hydrolysis of BCMs to smaller peptides in fermented dairy products (Jarmolowska 2012, Otte *et al.,* 2011and Atlan *et al.,* 1990)

Lactic acid bacteria posses' X-prolyl-dipeptidyl-aminopeptidase (PepX) activity may further hydrolysed BCM 7 to di or tri peptides (Donkor *et al*., 2007b). Muehlenkamp and Warthesen (1996) also reported that proteolytic system of *L. lactis* ssp. *cremoris* can degrade BCM 7 to half of its original concentration within 6–15 weeks of storage. De Noni and Cattaneo (2010) reported absence of BCM 7 in 35-days old Taleggio cheese prepared with lactic acid bacterial strains of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* as the starter culture. However number of studies reported the X-prolyl-dipeptidyl-aminopeptidase (PepX) activity of lactic acid bacteria in fermented dairy products (Atlan et al., 1990, Otte *et al*., 2011) but role of LAB or mould in formation of BCM 7 remains poorly understood. De Noni and Cattaneo (2010) and Sienkiewicz-Szłapka *et al*. (2009) also reported that the level of BCM 7 in Brie cheese and Rokpol cheese ripened with both mould and LAB are considerably higher than that in other cheeses ripened with LAB. Similarly, other authors have reported high levels of BCM 7 in Brie (Jarmolowska *et al.*, 1999) and in Blue cheese (Norris *et al.*, 2003). However, the link between β-CN A1 or β-CN A2 variants in the formation of BCM 7 in milk products currently has been received little attention. Almost all studies that have investigated BCM 7 and other related BCMs in commercial milk products did not characterize the type of β-CN variant of the raw milk or the products. However according to De Noni and Cattaneo (2010), both β-CN A1 and β-CN A2 variants are identified in cheeses where BCM 7 is present. Therefore, it will now be necessary to elucidate which variant is the source of BCM 7 during cheese making by future investigations on the occurrence of BCM7 and other related BCMs in cheese produced from milk of a single β-CN variant.

2.4.5 *In-vitro* digestive extract of milk and milk products

BCM 7 is mainly generated due to enzymatic hydrolysis of milk protein by gastrointestinal enzymes such as pepsin, trypsin and elastase (Fig. 2.8). A number of *in-vitro* studies reported that digestive extracts of milk and milk products are higher in BCM 7 concentration than in the raw milk (De noni and Cattaneo 2010; Cieślińska *et al*., 2012, Ul Haq *et al.*, 2015 and De Noni *et al.*, 2015). It is well known that pepsin and trypsin do not liberate BCM 7 from β-CN. Elastase cleaves the peptide bond between isoleucine (66) and histidine (67) (Jinsmaa and Yoshikawa 1999) and releases the carboxyl terminal of BCM-7. However pepsin is required to release the N-terminal amino acid of this peptide. Whereas, trypsin probably has similar hydrolytic action as of pepsin (Jakobsson *et al.*, 1983). Thus, the *in-vitro* hydrolysis of beta-casein with the mixture of the gastric intestinal enzymes can reflect a potential of forming of BCMs in the digestive tract.

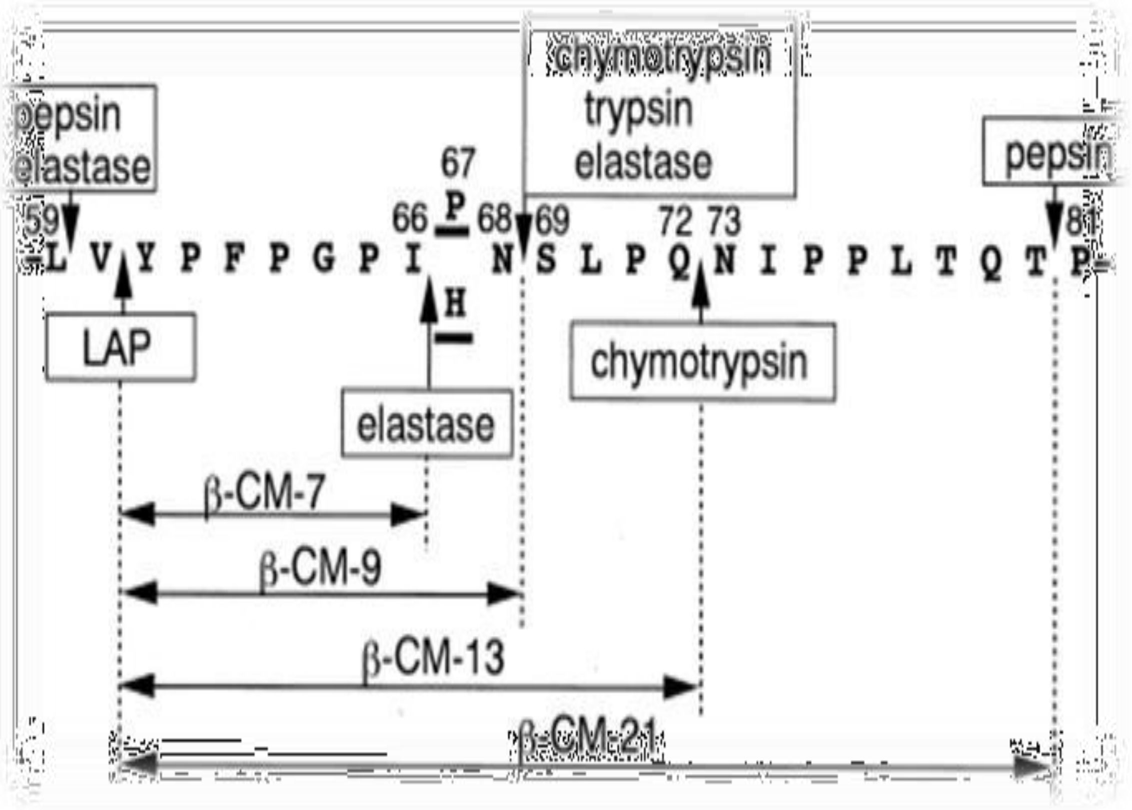


Fig. 2.8. Formation of Beta casomorphins peptides during enzymatic hydrolysis (Barnett *et al.*, 2014)

2.5 Digestion of proteins

In biological system digestion of protein starts in the stomach, where proteolytic enzyme pepsin breaks down the protein into smaller fragments of peptides. Firstly pepsin will act on the internal peptide bonds of ingested proteins, preferably at sites of hydrophobic and aromatic amino acids, such as tyrosine, phenylalanine and lysine (Rawling 2016). Pepsin shows optimum activity at pH 2.0 and loses its activity as pH increases and gets completely inactivated at pH 6.5 that is attained in the duodenum portion of the digestive system. When partially- digested peptide mixtures reach the duodenum, the intestinal enzymes starts hydrolyzing the semi-digested peptide and continue to break down the protein. Enzyme trypsinogen releases from the pancreas and is converted to the active form - tryspin via an enteropeptidase by proteolytic cleavage. Trypsin cleaves C- terminally to lysine and arginin. Chymotrypsin cleaves C-terminally to aromatic and bulky amino acids, and elastase cleaves C-terminally to alanin, valine and to some extent leucine (Rawling, 2016). Amino peptidase and carboxy peptidase cleavage the N and C terminal amino acid respectively. The pH in the duodenum is increased to 5-7.5, due to the release of pancreatic juices and bicarbonates. Alkaline condition inactivates the gastric enzymes, and gives the maximum activity intestinal enzymes. The final stage of digestion of proteins takes place on the surface of intestinal enterocytes, by brush boarder enzymes, where peptides are further hydrolysed to amino acids, di and tri-peptides (Verhoeckx *et al,.* 2015). Then amino acid, di and tri peptide are absorbed by the enterocytes of the jejunum and ileum, where further degradation takes place by intracellular proteases before entering in to the blood stream.

2.5.1. Standardized static *in-vitro* digestion method

In-vitro simulating digestion method is widely used to study the gastro-intestinal behavior of food. Although human nutritional studies are still being considered the "gold standard" for addressing diet related questions, *in-vitro* methods have the advantage of being more rapid, less expensive, less labor intensive, and do not have ethical restrictions. This allows a relatively large number of samples to be measured in parallel for screening purposes. Reproducibility, choice of controlled conditions and easy sampling at the site of interest make *in-vitro* models very suitable for mechanistic studies and hypothesis building. Simulated digestion methods typically include the oral, gastric and small intestinal phases, and occasionally large intestinal fermentation. These methods try to mimic physiological conditions *in-vivo*, taking into account the presence of digestive enzymes and their concentrations, pH, digestion time, and salt concentrations, among other factors.

CHAPTER –3

Materials & Methods

This chapter deals with the various materials and methodologies used during the period of research for identification of BCM 7 peptide from cross bred Karan Fries bovine, including equipments used during the present investigation relating to the technological,analytical and statistical aspects.

3.1 Materials

3.1.1 Procurement of milk

Crossbred Karan Fries (KF) (Tharparkar X Holstein Friesian) bovine breed selected for study were maintained at Livestock Research Centre of NDRI, Karnal. Segregation of Karan Fries to A1A1 and A2A2 genotype type was carried out on the basis of data provided by Proteomics Lab of Animal Biotechnology Center, NDRI and National Bureau of Animal Genetic and Resources (NBAGR). Animals were kept at same environmental condition and diet to avoid any difference. Twenty four healthy milking cows were selected, twelve from each genotype. Selected cows were free from anytype of health implication and any sign of mastitis. Raw milk of pure A2A2 and A1A1 genotypes having somatic cell count less than 100,000 cell/ ml and negative clot on boiling test (COB) used for further study to avoid any chance of mastitis effect.

3.1.2 Chemicals

Standard of BCM 7 peptide (purity >98.7%) was purchased from Sigma Aldrich. All other chemicals used in this research were of analytical grade from Sigma-Aldrich (India), Merck (India). Solvents and required chemicals were of HPLC grade. The reagents were made freshly as per the standard protocol. The ultrapure water was purified using milli Q system which was further filtered through 0.20 micron filter using vacuum filter. ELISA kit for BCM 7 (bovine) Cat. No. S-1334 was procured fromPeninsula Laboratories International, Inc., USA.

3.1.3 Glassware

Glasswares from Borosil were used for analysis. They were thoroughly cleaned using detergent solution, rinsed with running tap water followed by a rinse with distilled water and dried in hot air oven before analysis

3.1.4 Plastic ware

Plastic ware like centrifuge tubes, micro centrifuge tubes, storage vials and storage containers were procured from Tarsons Products Pvt. Ltd., Kolkata.

3.1.5 Equipments used during study

3.1.5.1 RP-HPLC 1200 Agilent HPLC system

3.1.5.2 Centrifuge high speed centrifuge 6500, KUBOTA

3.1.5.3 Labconco lyophilizer

3.1.5.4 Vacuum centrifuge Labconco Centrivap

3.1.5.5 ELISA READER Infinite 200Pro (Tecan i-control)

3.2 Standardization and validation of protocols for quantification of BCM 7 usingRP-HPLC

3.2.1 Preparation of BCM 7 standard solutions

Stock solution of BCM 7 (1 mg/ml) was prepared by dissolving 5 mg of standard peptide in a 5 ml of ultrapure water and kept into HPLC vials and stored -20^{0}C. Working stock solutions (100 μg/ml) of BCM 7 was prepared by diluting stock solutions in ultrapure water and acetonitrile (50:50). Serial standard solutions of BCM 7 in the range 0.2 -10 μg/ml and 10-100 μg/ml were prepared from the working stock solutions. The standard solutions were prepared freshly before everyday analysis. Standard working standards repetitively injected to HPLC system and response was observed.

3.2.1.1. RP-HPLC condition

Experiments were performed using 1200 Agilent HPLC system. Chromatographic separation of the analyte was carried out using a ZORBAX 300SB- C18 LC column (4.6 x 250 mm x 5μm) by injection of 20 μl of filtered extract at a flow rate of 0.5 ml/min. The column was protected by a Guard column in-line filter. The mobile phase consist of acetonitrile with 0.09% tri flouro acetic acid (TFA) (solvent A) and ultrapure water with 0.1% TFA (solvent B) in gradient flow. The chromatography runs start with 90% solvent A for 10 min, followed by gradual decrease in concentration of solvent A by 5% for every 10 min up to concentration of solvent A reached 70%. Then linear gradient was maintained for 30 min with solvent A and Solvent B concentration (70:30). After that, concentration of solvent A was further decrease by 10 % with every 10 min till the ratio of solvent A and B reached to (10:90). The chromatogram was recorded at 214, 220 and 280nm using DAD detector. The column temperature was maintained at isothermal condition for inlet and outlet that is 35^{0}C. To minimize potential carryover, the syringe used for injection was washed three times with acetonitrile and water (50:50) solvent before and after each injection.

3.2.2 RP-HPLC performance

Serial standard solutions were repetitively injected into the HPLC system to assess the performance of instrument. The determination of linear range was performed by analyzing standard solutions in concentrations from 0.2 to 1.0 μg/ml for solvent system (Fig. 3.1). Low concentrations of standard solutions were used for evaluation of instrument detection limit (IDL) and instrument quantification limit (IQL).

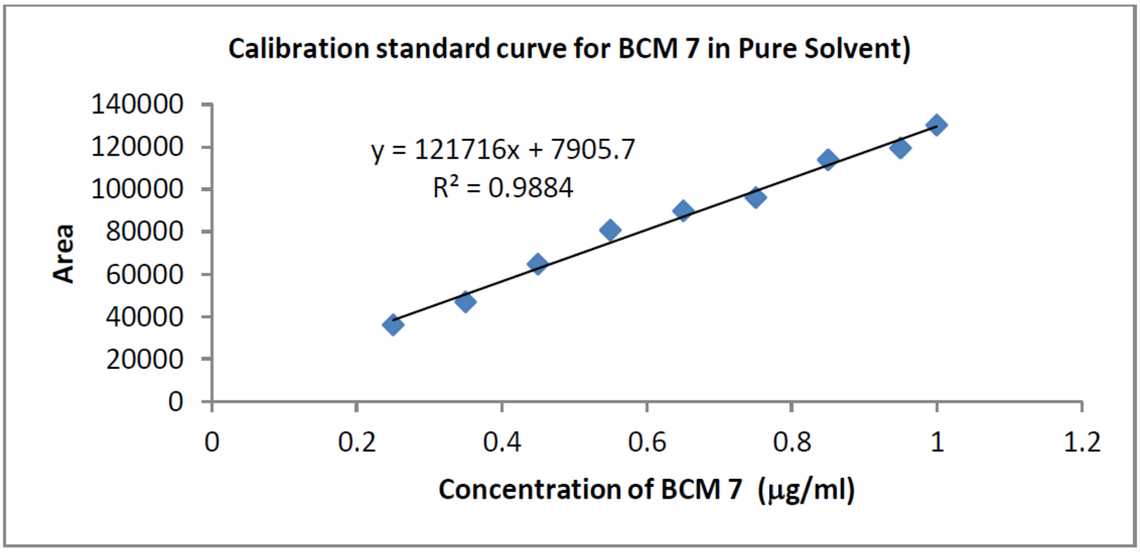


Fig. 3.1. Calibration standard curve for BCM 7 peptide in Acetonitrile and milli Q water (1:1)

3.2.3 Validation of the analytical method in milk system

Parameters of the HPLC analytical method that used to validate the developed methods were instrumental linearity, instrumental detection limits (IDLs) and instrumental quantification limits (IQLs), peak identification criteria (retention time and area), accuracy, precision, inter-day reproducibility, method limits of detection (LODs), method limits of quantification (LOQs) and matrix effects.

3.2.3.1 Preparation of calibration standard in milk system

3.2.3.1.1 Preparation of BCM 7 standard solutions in milk system

Milk sample from A2A2 genotype bovine was spiked with different label of BCM 7 (5- 100 μg/ml).

3.2.3.1.2 Extraction of BCM 7 from milk

Extraction of BCM 7 from milk samples was carried as procedure used for the extraction of water soluble peptides (Nguyen *et al*., 2014) with some modification. Following protocol as depicted in Fig 3.2 was used for the extraction of BCM 7 peptide from spiked milk.

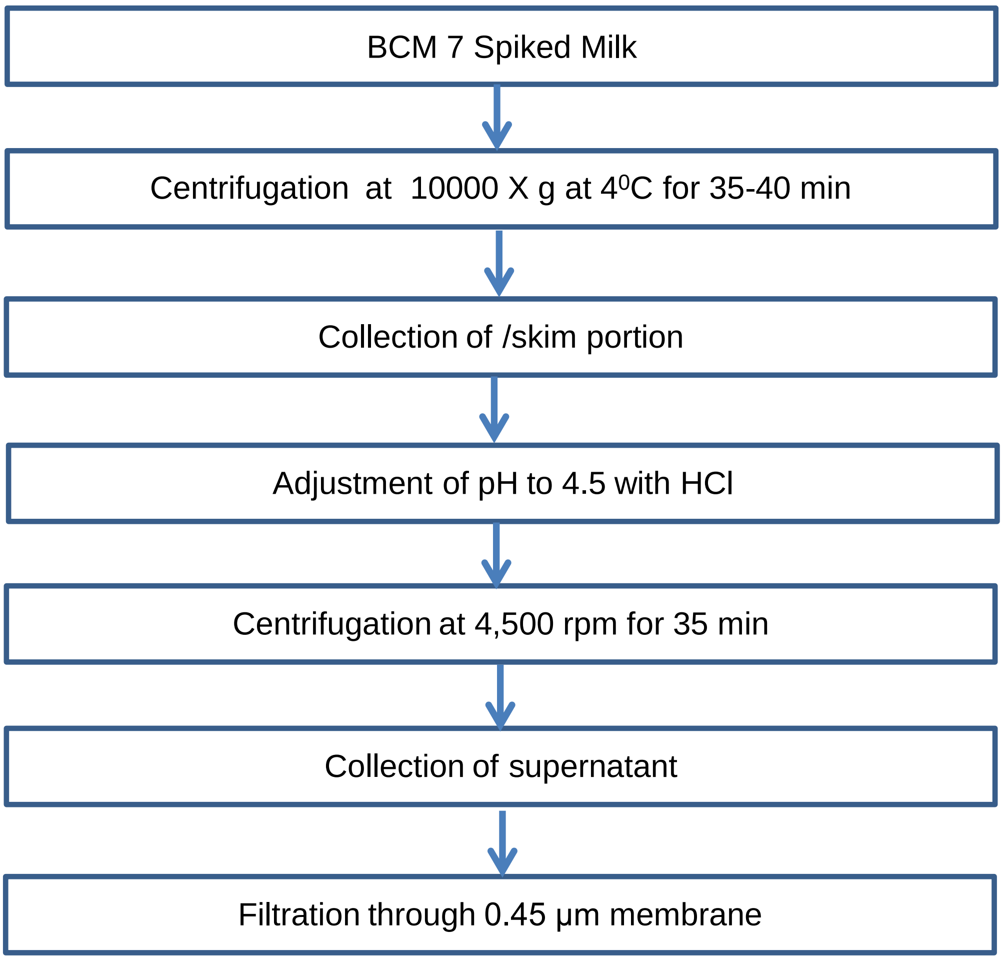


Fig. 3.2. Procedure for extraction of BCM 7 from spiked milk samples

3.2.3.1.3 Method limit of detection and limit of quantification

Method limit of detection and limit of quantification were assessed by recovery study in milk samples spiked with standard BCM 7 at the low level of 5 μg/ml. RP-HPLC chromatogram of milk spiked at different level were depicted in Figure 3.3 and linearity range were tested at six different levels of concentration (5, 10, 25, 50, 100 and 125 μg/ml) (Fig. 3.4).

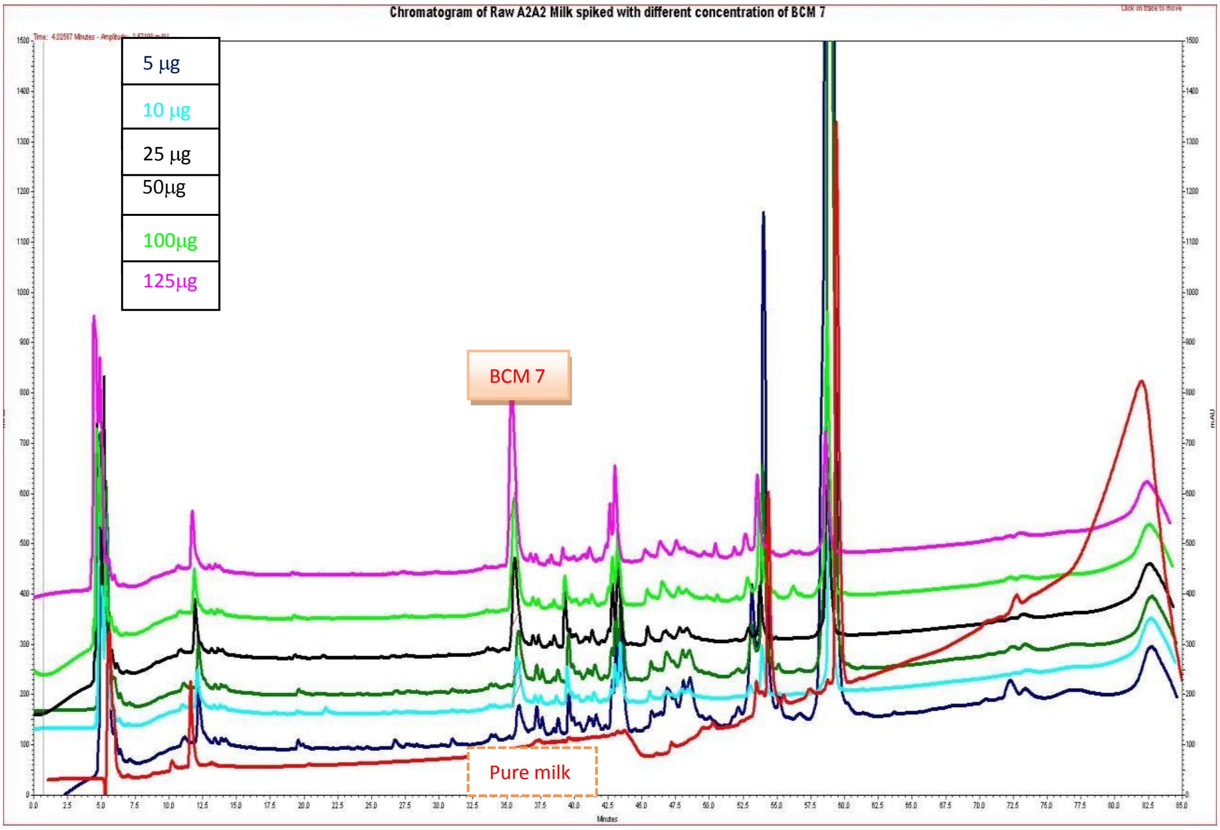


Fig. 3.3 RP-HPLC chromatogram of water soluble extract of milk spiked with different concentrations of BCM 7

Accuracy and precision were expressed as recovery relative standard deviation (%RSD), respectively. Pure A2 milk sample from A2A2 genotype cow was used as control for the study (Fig 3.3).

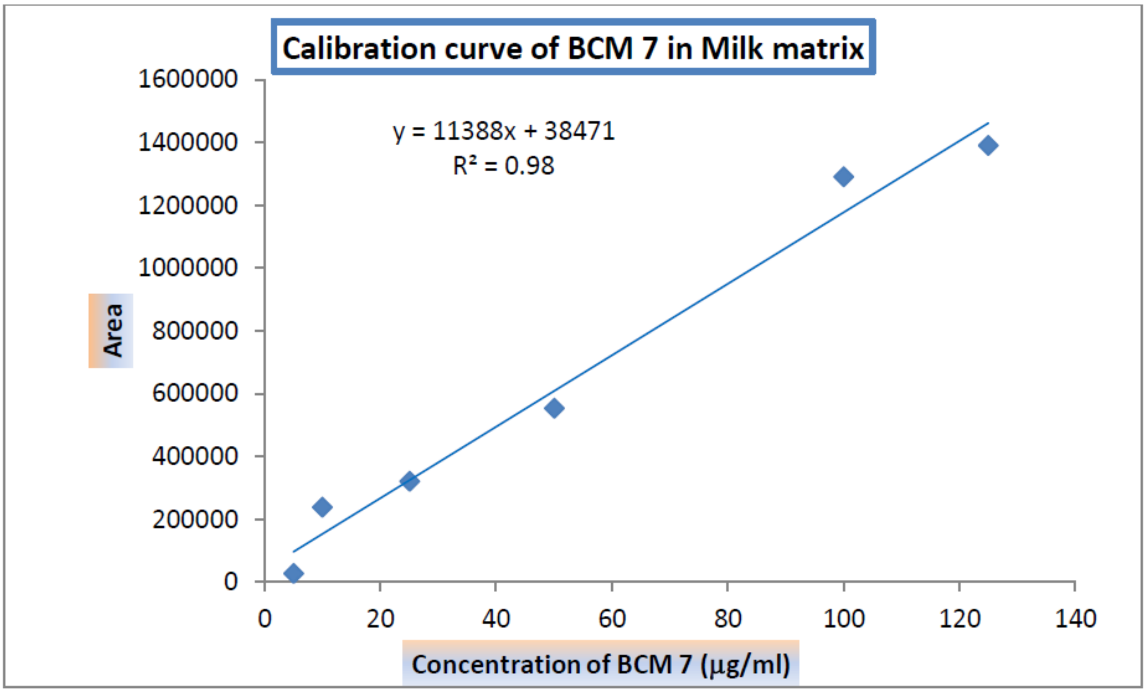


Fig. 3.4. Standard calibration curve of BCM 7 in milk matrix

3.2.3.1.4 Limit of detection and limit of quantification

The detection limits for BCM 7 were determined by reducing the concentration of injection. The limit of detection (LOD) and limit of quantification (LOQ) for standard were determined on the basis of signal to noise ratio. The detection limit is the injected amount that results in peak with a height at least three times as high as the base line noise level. The quantification limit is the injected amount that results in a peak with the height at least 10 times as high asthe baseline noise level.

For milk matrix, the LOD and LOQ were calculated by measuring standard deviation of the blank response.

The LOD may be expressed as:

LOD= 3.3 Ω / S

Where Ω = the standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The LOQ may be expressed as: LOQ= 10 Ω / S

3.2.3.1.5 Accuracy and precision of the developed method

The accuracy is calculated as percent recovery by the assay of known added amountof analyte in sample. Standard BCM 7 will be spiked in to the milk and milk products at different level of concentration.

3.2.3.1.6 Inter-day reproducibility

Inter-day reproducibility was determined by repeated analysis (n=2) on three consecutive week on three milk samples spiked with BCM 7 at four different levels. Inter-day reproducibility was expressed as %RSD.

3.2.3.1.7 Evaluation of matrix effect

The matrix effect was estimated by comparison of response of BCM 7 in pure solvent (acetonitrile and pure milli Q water) with that in milk system at same concentration. Thematrix effect was calculated using following formula:

$$\%\ \text{Matrix Effect} = \frac{\text{(Area of BCM 7 in ultrapure solvent-Area of BCM 7 in milk) matrix}}{\text{Area of BCM 7 in pure solvent}} \times 100$$

3.3 Standardization and validation of protocols for quantification of BCM 7 using ELISA assay

Serial dilution of BCM 7 standards (0.02 to 25.0 ng/ ml) were prepared as per theinstruction given in the ELISA kit. The extraction of BCM 7 for the assay was performed on C18 Solid phase extraction column (capacity 1g) using acetonitrile with 1% TFA and milli Q water as per the protocol provided in the kit. The protocol (I) mentioned in kit was used for the estimation of BCM 7 in milk samples. According to protocol 50 μl standards or samples (in diluents) were added with 25 μl of antiserum and 25 μl Bt- tracer in to each well of immunoplate. In blank well sample was replaced by 50 μl diluent. Immunoplate was incubated for 2 h at room temperature. Immunoplate was washed 5 times with 300 μl Enzyme Immuno Assay (EIA) buffer. Then 100 μl streptavidin-HRP was added in to each well and incubated for 1 h at room temperature. Again immunoplate was washed 5 times with 300 μl EIA buffer. 100 μl TMB solution was added in to each well and kept for 30-60 min as blue colour developed and absorbance was measured at 650 nm. Semi log standard curve was prepared using absorbance verses concentration of BCM 7 in standard solutions. Concentration of BCM 7 in milk samples was calculated from semi log graph (Fig. 3.5.).

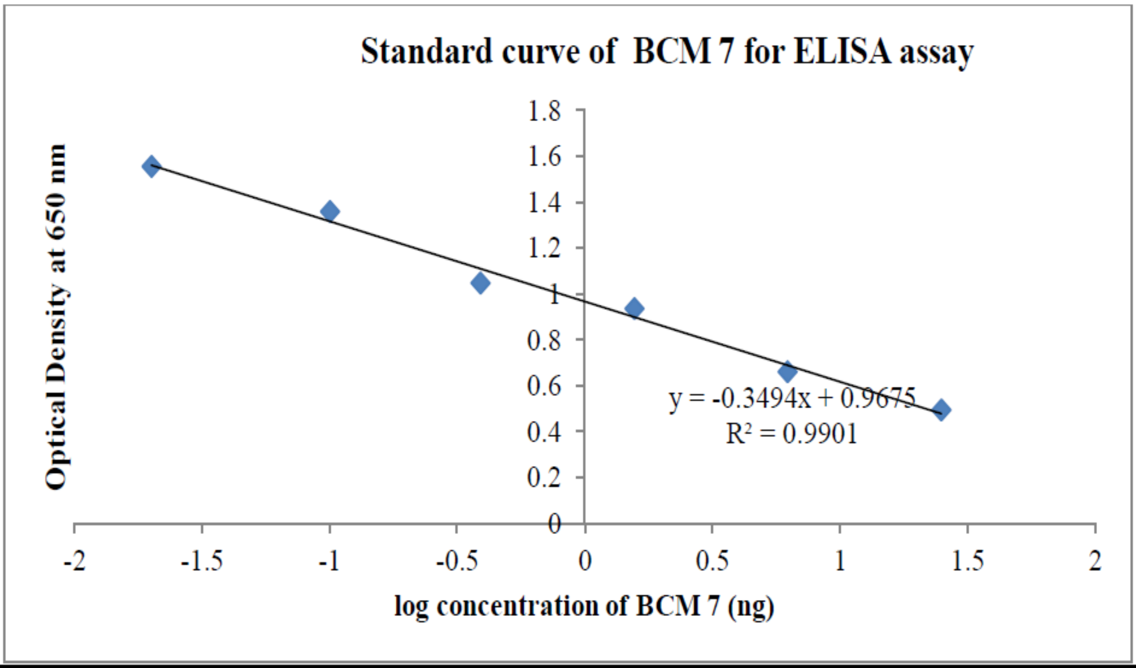


Fig. 3.5. Standard calibration curve for quantification of BCM 7 in milk samples using ELISA

3.4 Preparation of different milk products from milk of A2A2 and A1A1 genotype bovine

Raw milk (A1 and A2 type) of KF breed was procured from livestock centre. Different milk and milk products as shown in figure 3.6 were prepared as per standard protocol follows in the experimental dairy plant of NDRI, Karnal using different processing condition such as heating, acid coagulation, fermentation and combination of the processes.

3.4.1 Preparation of thermally processed milk from A1A1 and A2A2 genotype cow

3.4.1.1 Pasteurized milk prepared by heating raw milk at 63 ^{0}C for 30 min (LTLT).

3.4.1.2 Boiled milk prepared by boiling raw milk at boiling temperature for 5 min.

3.4.1.3. Sterilized milk prepared by heating raw milk at 121^{0}C for 10 min.

3.4.2 Preparation of *dahi* from A1 and A2 cow milk

3.4.2.1. Preparation of culture:

Culture used for preparation of *dahi* was procured from the experimental dairy, NDRI, Karnal. NCDC-167 Chr. Hansen's Lab, Denmark BD-4/CH-4 Culture was inoculated in sterilized milk (A2A2 and A1A1 genotype separately) at 37^0C for 24 h. After two successive inoculations in pure genotype milk, prepared cultures were furtherused for *dahi* preparation.

3.4.2.2. Preparation of *dahi*

Eight batches of *dahi* from each group were prepared according to standard procedure. Fresh milk from both genotypes were heated at 90 °C for 30 min, and cooled to 30 ± 2°C. Subsequently, cultures prepared in pure genotype were added to their respective milk at 1.50% (*w*/*v*) level. The mixtures were put into sterilized glass containers and incubated at 30 ± 2°C for 7-8 h. After incubation, *dahi* samples were stored at 5 ± 1 °C forone week.

3.4.3 Preparation of Cheddar cheese from A1 and A2 cow milk

3.4.3.1. Preparation of culture

Culture used for preparation of Cheddar cheese was procured from the experimental dairy, NDRI, Karnal. NCDC-167 Chr. Hansen's Lab, Denmark BD-4/CH-4 Culture was inoculated in sterilized milk (A2A2 and A1A1 genotype separately) at 37^0C for 24 h. After two successive inoculations in pure genotype milk, prepared culture was further used for preparation of Cheddar cheese.

3.4.3.2. Preparation of Cheddar cheese

Four batches of Cheddar cheese were prepared as per standard procedure from each A1 and A2 milk as showed in Fig. 3.6. Bovine chymosin (rennet) from Hansen (Denmark) was used as coagulating agent.

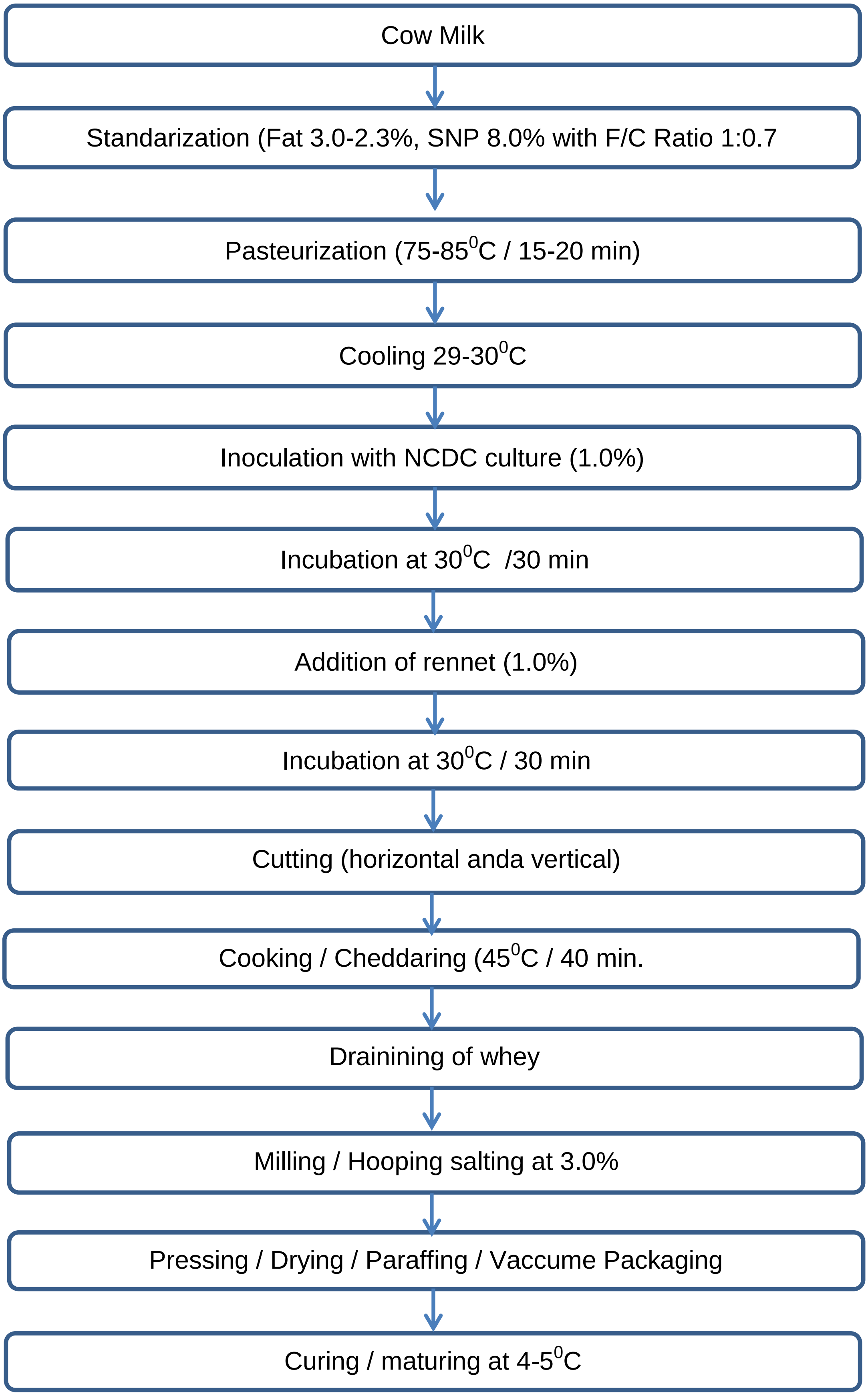


Fig. 3.6. Standard procedure for preparation of Cheddar cheese

3.4.4 Preparation of *Chhana* from A1 and A2 cow milk

Chhana was prepared with milk from A2A2 and A1A1 genotype cow using standard procedure (Kundu and De 1972) as depicted in Fig. 3.7.

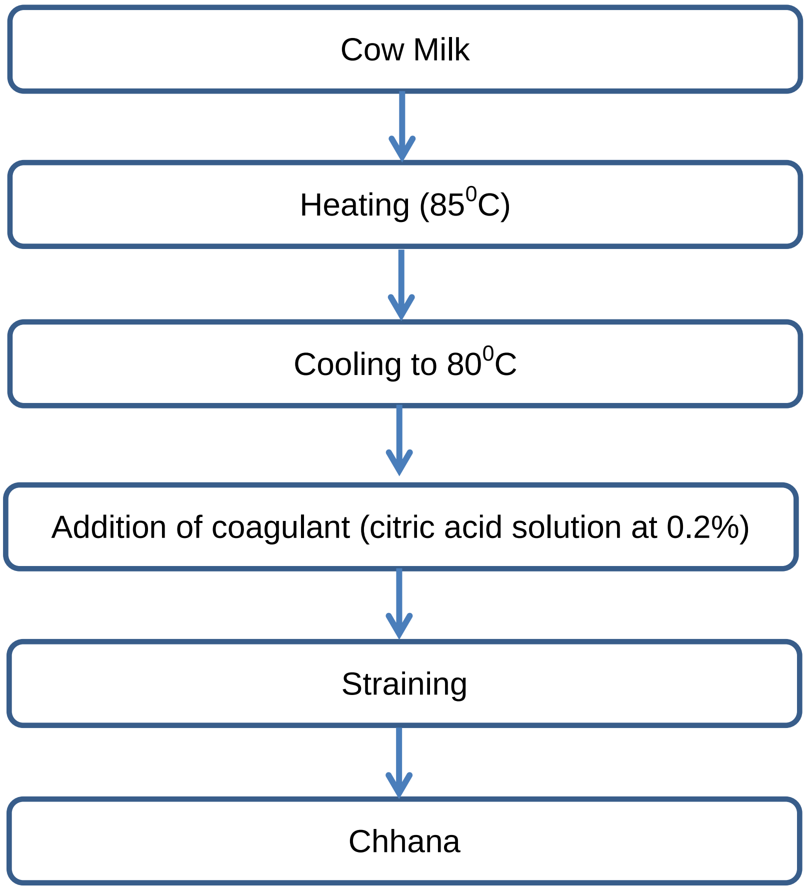


Fig. 3.7. Flow diagram for preparation of *Chhana*

3.4.5 Simulated *in-vitro* digestion of Milk, *Dahi,* Cheddar cheese and *Chhana* prepared from A1 and A2 milk

Digestibility study was carried out by using Static Simulated Gastro-Intestinal Digestion (SGID) Model (Minekus *et al.,* 2014) (Fig. 3.8). Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) used in study are made up of the corresponding electrolytes stock solutions, enzymes, $CaCl_2$ and water.

Simulated *in-vitro* gastric model consist of following phase-

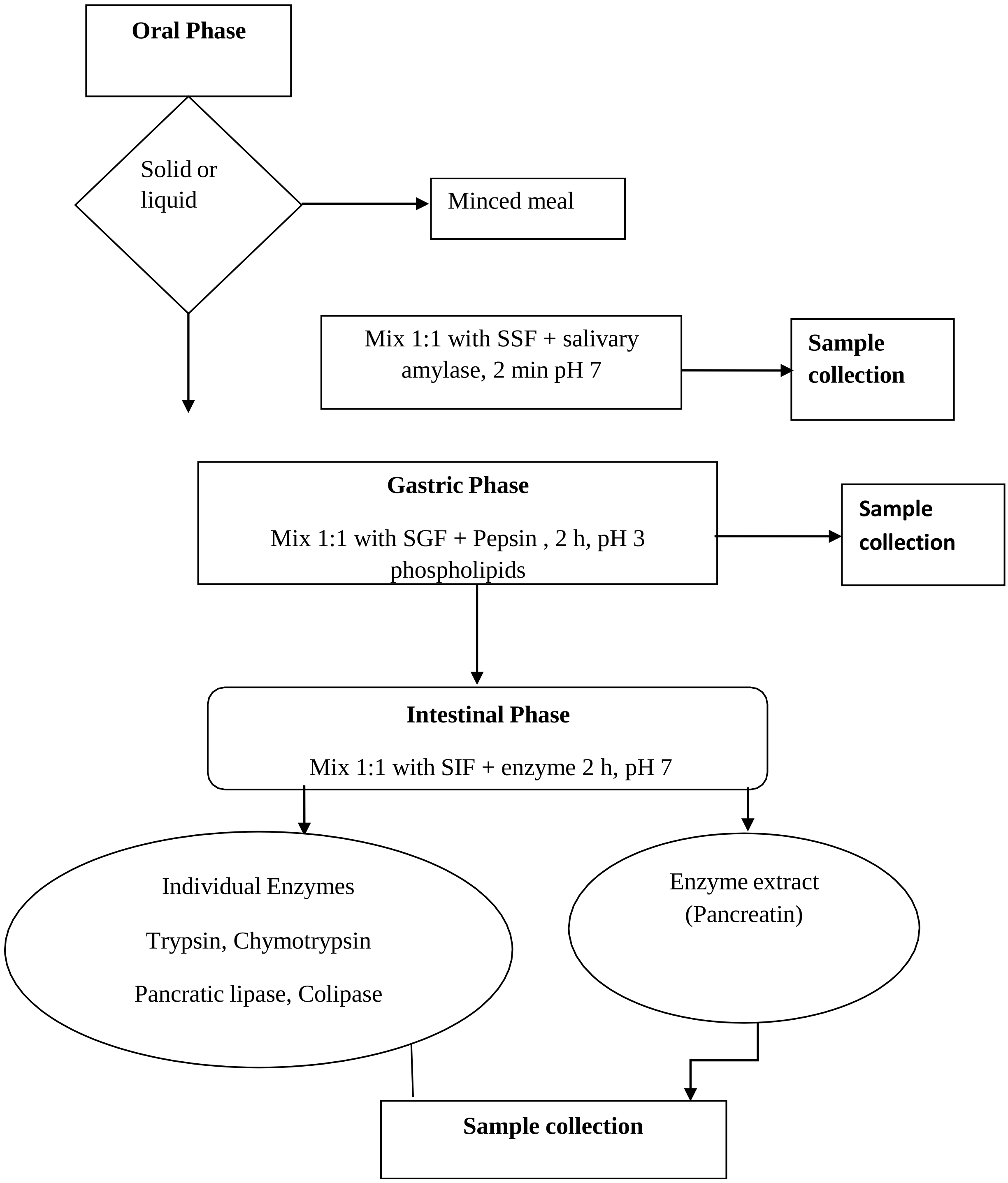


Fig. 3.8. Simulated *in-vitro* digestion protocol used in the study

3.4.5.1. Oral phase

Mastication of solid food was simulated by mincing an appropriate amount of food using a commercially available manual or electric mincer, or similar, commonly used in kitchens to mince the dairy products. Simulates Salivary Fluid (SSF) electrolyte stock solution was added to create a thin paste-like consistency. A final ratio of food to SSF of 50:50 (w/v) was maintained. Human salivary α-amylase was used in this study. Recommended time of contact with the enzyme was 2 minutes at 37°C.

3.4.5.2. Gastric phase

Liquid food after the oral phase was exposed to gastric phase. Final ratio of food to Simulated Gastric Fluid (SGF) maintained at 50:50 (v/v) after addition of other recipients and water. Porcine pepsin was used to final digestion mixture. 1M HCl was added to reduce the pH to 3.0. The recommended time used for digestion was 2 hours at 37°C with occasional shaking.

3.4.5.3. Intestinal phase

In this phase five parts of gastric chyme was mixed with 4 parts of Simulated Intestinal Fluid (SIF) electrolyte stock solution to obtain a final ratio of gastric chyme to SIF of 50:50 (v/v) after additions of other recipients and water. 1 M NaOH was added to neutralize the mixture to pH 7.0. Digestive enzymes were added aspancreatin from porcine pancreas.

3.4.5.4. Sampling during digestion

The sample of digested material were collected at each stage of digestion such as after oral, gastric and intestinal phase and subjected to BCM 7 analysis.

In Table 3.1. all information about the composition of digestion mixture used at each stage of digestion during study were given.

Table 3.1: Recommended concentration of electrolytes in SSF, SGF and SIF

Constituents		SSF pH 7.0	SGF pH 3.0	SIF pH 7.0
	Electrolytes (g/L)	**Stock (ml)**	**Stock (ml)**	**Stock (ml)**
KCl	37.3	15.1	6.9	6.8
NaCl	117.0	-	11.8	9.6
KH_2PO_4	68.0	3.7	0.9	0.8
$NaHCO_3$	84.0	13.6	25	42.5
$MgCl_2(H_2O)_6$	30.5	0.15	0.1	0.33
$(NH_4)_2CO_3$	48.0	0.06	0.5	-.
$CaCl_2(H_2O)_2$	44.1	1.5	0.15	0.6
	For pH adjustment			
NAOH		-	-	-
HCl		1.1	15.6	8.4
	Enzymes (U/ml)			
Salivary amylase		75	-	-
Pepsin		-	2000	-
Pancreatin		-	-	100
Trypsin		-	-	100
Chymotrypsin		-	-	25
Pancreatic Lipase		-	-	2000
Colipase		-	-	4000
Pancratic amylase		-	-	200
Bile		-	-	10 mM

3.5 Preparation of fraction rich in BCM 7 from milk and milk product and theirdigestive extracts

3.5.1 Extraction of BCM 7 from milk products

Similar procedure as given in **3.2.3.1.2** for extraction of BCM 7 from undigestedand oral phase milk and milk products (Fig 3.2).

3.5.2 Extraction of BCM 7 from digested samples

The water soluble extract of digestive mixture of gastric and intestinal phase were obtained by centrifugation at 4,500 rpm for 35 min at 5^0C and filtering the supernatant fluid through a whatman No 41 filter. The filtrate will be further subjected to ultra filtration through 3kDa membrane, followed by freeze drying.

3.6 Proximate compositional analysis of milk

Compositional analysis of raw milk was carried out as per AOAC (2000) and FSSAI (2016) to study any differences between A1 and A2 genotypes.

3.6.1 Determination of moisture

The sample was dried to constant weight at 102 ± 2°C and the loss in weight reported as moisture. The moisture content was done by IS 11623:1986 method.

3.6.1.1 Material

1) Flat bottom moisture dishes with cover stainless steel, 50 mm diameter and 25 mm dept.
2) Hot air oven : Maintained at 102 ± 2°C
3) Desiccator

3.6.1.2. Procedure

Approximately 10 gm of sample was taken in previously weighed moisture dish. Kept in oven for 1 hrs for drying. After 1 h weight was taken and again kept for drying for ½ h cooling and weighing until constant weight achieved.

3.6.1.3. Calculation

$$\text{Moisture \% by mass} = \frac{(M_1 - M_2)}{(M_1 - M)} \times 100$$

Where,

M = Mass of empty dish in gm

M_1= Initial mass in gm before drying in gm

M_2 = Final mass after drying in gm

3.6.2 Determination of protein by Kjeldahl method

3.6.2.1. Pre treatment

1 g digestion mixture was transferred to a clean and dry digestion tube. 2 gm of test sample was weighed to the nearest 0.1 mg in to the digestion tube. 10 ml of sulphuric acid was added along the sides of the digestion tube. The content of the tube were mixed and then left to stand for 10 min.

3.6.2.2. Digestion

The digestion block was set at a low initial temperature so as to control foaming (approximately 180°C). The tube was transferred to the digestion block and the exhaust manifold was placed, which was itself connected to a water jet pump on the tube. The suction rate of the water jet pump was sufficient to remove fumes. The sample was digested until white fumes developed. The temperature of digestion block was then increased to 411°C and digestion of the sample was continued until the digest was clear. The digestion was completed in 40 to 60 min. The tube was removed from the block with the exhaust manifold in place and allowed to cool for at least 15 min. Once the tubes were sufficiently cool to handle, the exhaust manifold was removed and 50 ml of water was carefully added to each tube.

3.6.2.3. Distillation

The digestion tube was transferred to the distillation unit and programme for automatic distillation was run. The conical flask containing 50 ml of boric acid solution was placed under the outlet of condenser in such a way that the delivery tube was below the surface of the excess boric acid solution. The distillation unit was adjusted to dispense 40 ml of sodium hydroxide solution to distill off the ammonia. The distillate was collected in the excess boric acid solution.

3.6.2.4. Titration

The contents of the conical flask were titrated with 0.1N hydrochloric acid standard volumetric solution using a burette and the amount of titrant used was readout. The end point was indicated at the first appearance of violet colour in the contents.

3.6.2.5. Blank test

A blank test was carried out following the procedure as described above using 2ml of water and about 0.2 gm of sucrose instead of test portion.

3.6.2.6. Calculation

The nitrogen content (Wn) was calculated by using the following equation

$$Wn = \frac{1.4007 \times (Vs - Vb) \times N}{m}$$

Where,

Wn = Nitrogen content of the sample, expressed as percentage by mass

Vs = Numerical value of hydrochloric acid standard volumetric solution used indetermination in ml, expressed to the nearest 0.05 ml

Vb = Numerical value of the volume of hydrochloric acid standard volumetric solutionused in the blank test in ml, expressed to the nearest 0.05 ml

N = Numerical value of the exact normally of the hydrochloric acid standard volumetricsolution expressed to four decimal places

m = Numerical value of the mass of the test portion in gm expressed to the nearest 1mg

The crude protein content (Wp) was calculated using the following equation

$$Wp = Wn \times 6.38$$

Where,

Wp = Crude protein content, expressed as a percentage by mass

Wn = Nitrogen content of the sample, expressed as a percentage by mass to four decimal

6.38 = factor to express the nitrogen content as crude protein content

3.6.3 Determination of fat

Fat content of powder was determined by AOAC (2000)

3.6.3.1. Procedure

1 gm of sample was weighed in a Mojonnier fat extraction tube followed by addition of 9 ml of water (65 ± 5°C) distilled water and 1.25 ml of concentrated ammonia and then mixed in lower bulb of extraction tube, followed by addition of 10 ml ethyl alcohol and 25 ml diethyl ether. The tube was closed tightly and the content were vigorously shaken. The tube was then opened and 25 ml of light petroleum ether was added followed by vigorous shaking. The ethereal layer was decanted into previously dried, cooled and weighed dish. Extraction and decantation operation was repeated twice by using 15 ml diethyl ether and 15 ml petroleum ether. The solvent was dried in an oven at 100°C for 1 hr, finally the dish was cooled and then weighed.

3.6.3.2. Calculation

$$\text{Fat (\% by weight)} = \frac{W_1}{W_2} \times 100$$

Where,

W_1 = Weight in gm of residue after drying

W_2 = Weight in gm of sample

3.6.4 Determination of ash

3.6.4.1 Apparatus

1) Platinum or silica crucible : About 70 mm diameter and 25 to 5 mm deep.

2) Muffle furnace : Capable of being controlled at 550 ± 20°C.

3) Desiccator : Containing an efficient desiccant.

4) Safety tongs having long arms.

5) Bunsen burner or electric hot plate.

3.6.4.2. Procedure

Weigh accurately about 3 gm of the dried emulsion sample in the crucible, previously dried in a hot air oven and weighed. Heat the crucible gently on a burner or hot plate at first and then strongly in a muffle furnace at 550 ± 20°C till gray ash was obtained. Cool the crucible in a desiccator and weigh it. Heat the crucible again at 550 ±20°C for 30 min. Cool the crucible in a desiccator and weigh. Repeat this process of heating for 30 min, cooling and weighing until the difference between two successiveweighing was less than 1 mg .

3.6.4.3. Calculation

$$\text{Total ash (on dry basis), \% by mass} = \frac{M_2 - M}{(100 - M_0) \times (M_1 - M)} \times 100$$

Where

M2 = mass in gm, of the crucible with ashM = mass in gm, of the empty crucible

M1 = mass in gm, of the crucible with the material taken for the test

M0 = moisture, % by mass, calculated as per the method for dried emulsion powder

3.6.5 Titrable acidity

Titrable acidity of milk products was determined as per the method described inIS:1479 (Part I) (1960).

3.6.5.1 Procedure

10 gm of the yoghurt sample was weighed accurately into a 250 ml beaker and 30 ml warm water was added to it. 1 ml of phenolphthalein indicator solution was addedto the sample and the solution was then titrated against 0.1N sodium hydroxide solution.It was stirred vigorously throughout the titration and the titration was completed within20 sec. Another beaker containing 10 ml of

sample with 30 ml lukewarm water was kept as blank for comparison of colour. The persistence of pink colour for 30 sec indicated the end point.

3.6.5.2. Calculation

$$\text{Titratable acidity (\% LA)} = \frac{9\ AN}{W}$$

Where,

A = Volume in ml of sodium hydroxide required for titrationN = Normality of standard sodium hydroxide solution

W = Weight in gm of yoghurt used for the test

3.6.6 pH

pH of the milk and *dahi* was determined electrometrically with a pH meter using the method as described in IS: (SP:18,Part XI) (1981). The pH meter was first calibrated using standard buffer of pH 4 and 9.2 and standardized using pH buffer of 7 at 20 ± 0.1°C.

3.7. Degree of Hydrolysis

Degree of hydrolysis was evaluated by measuring the release of free amino acid residue using 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) assay (Adler-Nissen, 1979). The free amino groups were calculated against the L-leucine standards. The higher absorbance at 340 nm indicates a higher amount of free amino groups.

3.7.1 Reagents

3.7.1.1. TNBS working solution (0.1% w/v)

A fresh working solution of TNBS was prepared prior to assays by diluting the5% TNBS solution in 0.1M sodium bicarbonate aqueous solution

3.7.1.2 Sodium phosphate buffer (0.1 mmol/l pH 8.2)

3.7.2. Procedure

5 ml sample was mixed with the 1% TCA followed by centrifugation at 4500 rpm for 30 min at 4^{0}C and supernatant was filtered through whatman filter paper No 41. 50 μl diluted filtrate were mixed with 400μl sodium phosphate buffer (0.1 mmol/L, pH 8.2) and 400 μl 0.1% TNBS solution (prepared fresh in

same buffer). After 60 min incubation at 50^0C, reactions were stopped by adding 800µl 0.1mmol/ L HCL. The absorbance was measured at 340 nm using UV-spectrophotometer. A calibration curve was prepared using L-leucine in range of 0.2-2.0 mmol/L and results were expressed as mmol/L of leucine equivalents.

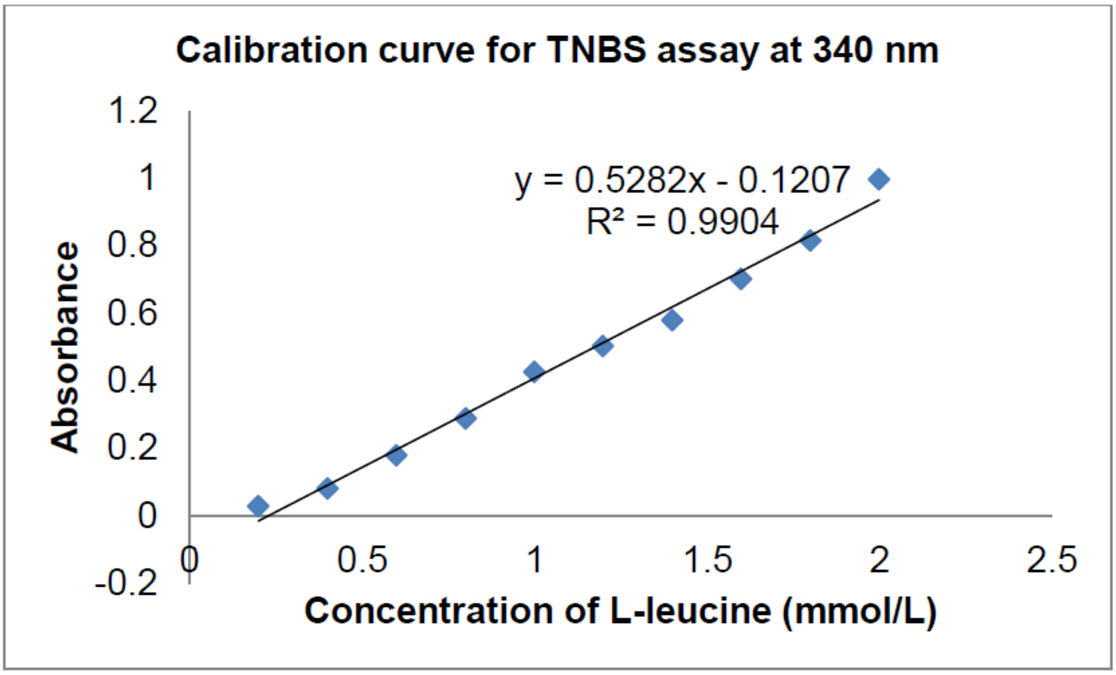


Fig. 3.9. Standard calibration curve of L-leucine for the determination of Degree of Hydrolysis

3.8 Estimation of PepX activity of *dahi* prepared from A1 and A2 milk during storage

X-prolyl-di petidyl aminopeptidase (PepX) enzyme is a unique characteristic of lactic acid bacteria, responsible for the degradation of proline rich peptides to tri, di or free amino acid residue. Hence PepX activity is important parameter for fermented product.

3.8.1 Preparation of extract

The extraction was carried out as mentioned by Nguyen *et al*., (2014). 20 g of *dahi* sample was thoroughly mixed with 1ml of 1 M sodium citrate. The tube was centrifuged at 4500 rpm for 30 min at 4^0C. The supernatant was separated and used for analysis.

3.8.2 PepX assay

An analysis of the Prolyl aminopeptidase X (PepX) activity in *dahi* samples was carried out (Miyakawa *et al*., 1991) using chromogenic substrate Gly-Pro-ρ-NA. Substrate was dissolved in 0.1M NaH_2PO_4-NaOH buffer of pH 6.5. Substrate and sample extract were separately incubated at 37^0C for 10 min. 0.5 ml of substrate was mixed with the 0.5 ml of sample extract and incubated at 37 °C for 10 min. The reaction was terminated by addition of 1 ml acetic acid (30% *v*/*v*). The released ρ-nitroanilide was monitored by measuring the absorbance at 410 nm using double beam spectrophotometer. The enzyme activity was calculated as unit μmol of ρ-nitroanilide released per min per ml of extract under the above assay conditions using molar extinction coefficient (8800 $mol^{-1}cm^{-1}$) nitroanilide at 410 nm (Malone *et al*., 2003).

$$\text{Enzyme activity} = \frac{\text{(Total volume of substrate × Change in absorbance per min)}}{\text{molar extinction coefficient × volume of sample}}$$

3.9 Determination of Salt content

Salt content in Cheddar cheese was determined by procedure laid down in IS 2785:1979

3.9.1 Procedure Weigh 10 g of prepared sample of Cheddar cheese in Erlenmeyer flask. Add 10 ml distilled water and 25 ml of 0.05 N silver nitrate solution. Warm the content at 75-80^0C . Add 10 ml of conc. Nitric acid and boil the content. Add 10 ml of iron alum solution and 50 ml distilled water. Titrate with 0.05 N potassium thiocyanate till appearance of orange ting.

3.9.2. Calculation

% salt	=	0.292/(V1-V2)/M
V1	=	volume of standard potassium thiocyanate equivalent to 25 ml silvernitrate
V2	=	volume of potassium thiocyanate used in the titration of excess silver nitrate
M	=	weight of cheese

3.10. Ripening Index in Cheddar cheese

3.10.1 Nitrogen in water soluble extract (WSN)

Water soluble extract (WSE) of the cheese sample was prepared as per the procedure described by Nguyen *et al*., (2014) with some modification. 10 g of grated cheese sample was taken and mixed with 100 ml of distilled water. Mixture was heated to 40^0C for 30 min and mixed thoroughly using Philips mixture. Centrifugation was carried out at 4500 rpm for 30 min at 4^0C, followed by filtration using Whatman filter paper. Filtrate was taken for estimation of water soluble nitrogen.

3.10.2. TCA soluble nitrogen (TCASN)

The TCA soluble nitrogen fraction was prepared by adding equal part of 24% TCA to WSE (Topcu and Saldamli, 2006). The mixture was allowed to stand for 30 minutes at 20°C and filtered through Whatman No. 42 filter paper. The nitrogen content was determined using Kjeldahl method.

3.10.3. Calculation

Ripening Index (RI) for checking primary proteolysis RI was calculate usingfollowing formula

RI = (Water Soluble Nitrogen / Total Nitrogen) x 100

To determine the secondary proteolysis % TCA SN/TN was calculated.

3.11. Microbiological characterization

Microbial analysis of yoghurt was performed using the method described in FSSAI (2016).

3.11.1. Dilution blank for *dahi*

The dilutions blank for *Dahi* were made from normal saline (0.80-0.85% NaCl) solution. For 1:10, 1:100, 1:1000 dilutions, 9 ml of test solution was poured in test tube. The mouth of the test tubes was closed with cotton plug. Test tubes containing dilution blanks were then sterilized at 15 psi (121°C) for 15 min in an autoclave. 1ml of thoroughly mixed sample aseptically transferred into dilution tubes containing 9 ml of dilution blank. The content of the test tubes were mixed well and this gave a dilution 1:10. From this initial dilution further dilution were prepared by transferring 1 ml into 9 mlblanks.

3.11.2. Standard Plate count/ Total plate count

Standard plate count was assessed by plating method using nutrient agar media.

3.11.3. Coliform

VRBA was used to enumerate Coliform count. 41.53 gm of VRBA agar was suspended in 1000 ml of distilled water and boiled to dissolve completely. The medium was boiled instead of autoclaving as suggested by manufactures.

3.11.3.1. Plating for Coliform

Diluted sample from suitable dilution (1 ml) was transferred to each of the petriplate in triplicate, 10-15 ml of the melted agar (at 45°C) was then poured and the contents were mixed well by rotating in a horizontal position. The content was allowed to solidify. The plates were then inverted and incubated at 37°C for 24 hrs.

3.11.4. Yeast and Mould

PDA was used to enumerate the yeast and mold counts in yoghurt. 39 gm of potato dextrose agar was suspended in 1000 ml distilled water and boiled to dissolve the medium completely. It was then filled in flasks and sterilized by autoclaving at 15 Ibs pressure (121°C) for 15 min. The pH of the medium was adjusted to 3.5 at the time of plating by using sterile 10% tartaric acid.

3.11.4.1. Plating for Yeast and Mould

Diluted sample from suitable dilution (1ml) was transferred to each of the petriplate in triplicate, 10-15 ml of the melted agar (at 45°C) was poured and the content were mixed by rotating in a horizontal position. The contents were allowed to solidify. The plated were inverted and incubated at 25°C for 2 days.

CHAPTER –4

Results and Discussion

Beta casomorphin 7 (BCM 7) is an opioid peptide consisting of seven amino acid residues, ranging from 60 to 66 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) of parent β-casein (β- CN). It is hypothesized that BCM 7 generally released during enzymatic hydrolysis of milk from A1A1 genotype bovine (Jinsmaa and Yoshikawa 1999) or dairy processing (Brooke-Taylor *et al*., 2017). Epidemiological studies have been reported by various researchers and suggested that BCM 7 and BCM 7 like peptides may contribute a range of chronic diseases (Thiruvengadam *et al*., 2021). There are certain studies available which provide the evidence that release of BCM 7 not only linked to milk containing A1 variant of β-CN but also identified in milk containing other variants of β-CN (Cieślińska *et al*., 2007, Cieślińska *et al*., 2012; Nguyen *et al*., 2014; Nguyen *et al*., 2015, Asledottir *et al*., 2017, Asledottir *et al*., 2018; Nguyen *et al*., 2019; Lambers *et al*., 2021).

Heat processing and fermentation are major processing techniques employed in preparation of milk products. Heat treatment not only destroys pathogenic and spoilage microorganism but also causes change in physiochemical properties of milk, especially due to denaturation of whey proteins. Then denatured β-lacto globulin gets attached to casein micelles which affects the physicochemical characteristics of milk and milk products. Thereby kinetics of proteolysis may be different in heat processed milk and milk products and may affect the formation and degradation of BCM 7 and its precursors. Fermentation of milk in to different products preparation involves a number of microorganism especially lactic acid bacteria, moulds etc. The microorganism used in preparation determines the proteolytic profile of milk protein along with condition of incubation, pH, and duration of storage. A number of studies reported that on storage concentration of BCM 7 decreases.

Acid precipitation is other common processing method used for the preparation of acid coagulated dairy products such as *paneer, Chhana*, cottage cheese. During manufacturing of such products formation of large structural aggregates of casein from normal colloidal dispersion of discrete casein micelles takes place, in which milk fat and coagulated serum proteins are entrapped together with whey. On addition of coagulating agent (citric acid) pH of milk is drops and causes progressive removal of tri calcium phosphate and calcium hydrogen caseinate from the surface of casein and its conversion into mono calcium phosphate and soluble calcium salt and free casein. Thereby colloidal particles become isoelectric and milk system is no longer stable hence casein gets precipitated in the coagulum. These all changes may affect the release of BCMs in dairy products.

No systematic studies are available related to processing treatments and BCM 7 formation or degradation. Whether BCM 7 does form at various processing steps or degrades requires further investigation. Therefore the aim of the study is to investigate the effect of different dairy processing condition on the formation and degradation of BCM 7.

In this study, an attempt has been made to standardize RP-HPLC coupled with UV- detector for quantification of BCM 7 in milk system for routine analysis. This successful validated method has been applied for the identification and quantification of the BCM 7 peptide in milk and milk products and their *in-vitro* digestive extracts to study the effect of different dairy processing condition on generation or degradation of BCM 7. The results obtained by RP-HPLC methods were cross checked by ELISA assay. Simultaneously peptide sequencing analysis was carried out using LCMS/MS for the authentication of BCM 7 in milk products by collecting the representative BCM 7 fraction from RP-HPLC analysis.

4.1. Standardization and validation of RP-HPLC method for the identification of BCM 7 in milk and milk products

Reversed-phase high performance liquid chromatography (RP-HPLC) is commonly used method for the identification of peptides in dairy products. In RP-HPLC method, RP-HPLC coupled with ultra violet (UV) detector was used in various studies for quantification BCM 7 in milk and milk products (Muehlenkamp and Warthesen, 1996; Jarmolowska *et al*., 1999; Cieślińska *et al*., 2007).

4.1.1. Optimization of RP-HPLC condition for quantification of BCM 7 in milk and milk products

The standard solutions of BCM 7 at lower level (0.25 μg/ml and 1.0 μg/ml) were injected in HPLC and data was recorded at 214, 220 and 280 nm. Solution of

BCM 7 was showing maximum absorption at 214 followed by 220 and 280 nm. The signal to noise ratio was highest at wavelength 220 nm (>100), hence 220 nm was finalized for the study. The linear range was tested using calibration standard curve as shown in Fig 3.1 and Regression coefficient (R^2) for BCM 7 was 0.9884, indicating good linearity in the tested range. Instrument LOD and LOQ were 0.03 and 0.09 μg/ml respectively at retention time of 36.35 ± 0.10 min**.**

4.1.2 Method validation for quantification of BCM 7 in milk system

For method validation, calibration standards were prepared in milk matrix. Procedure mentioned in Fig. 3.2 was used for the extraction of BCM 7 from milk. Chromatogram was recorded at 200 nm for different known concentration of BCM 7 depicted in Fig 3.3. The linear range was tested between 5.0 μg/ml to 125.00 μg/ml using calibration standards prepared in milk matrix (Fig. 3.4). Regression coefficient (R^2) was 0.98, indicating good linearity in tested range. Method LOD and LOQ for BCM 7 were 6.06and 20.205 μg/ml respectively with retention time of 36.44 ± 0.017.

4.1.3. Accuracy and precision of standardized method for the quantification ofBCM 7 in milk system

Milk samples were spiked with 4 different known concentrations of BCM 7 and extraction of target analyte was carried out in triplicate. The accuracy of the method was 90.00-103.00 % with 1.2 to 7.8 % precision in terms of relative standard deviation (Table 4.1). Nguyen *et al*., (2014 and 2019) also reported similar type of validated protocol for quantification of BCM 7 in yoghurt and milk samples with 103.00-109.00% accuracy and 1.00-7.00 % relative standard deviation

Table 4.1. Recovery and precision study for validation of standardized method for the quantification of BCM 7 in milk system

SpikedLevel (μg/ml)	Estimated concentration (μg/ml)(n=2)	Recovery (%)	Precision (%RSD)
20	18.17 ± 1.40	90.87	7.40
25	24.74 ± 0.029	98.97	7.84
100	108.11 ± 4.029	108.17	7.37
200	207.27 ± 2.489	103.50	1.20

(Values are mean ± SD)

4.1.4. Inter-day reproducibility of the standardized method for the quantification of BCM 7 in milk system

To assess the reproducibility of analytical method, repeated measurements of BCM 7 in spiked milk at 4 different levels were carried out on three consecutive days (Table 4.2). Relative standard variation of analytical method was 0.79 to 3.76 % indicating good inter-day reproducibility in tested range.

Table 4.2. Inter-day reproducibility of the standardized method for the quantification of BCM 7 in milk system

SpikedLevel (μg/ml)	Week 1(n=2)	Week 2(n=2)	Week 3(n=2)	Precision (%RSD)
	Estimated concentration (μg/ml)	Estimated concentration (μg/ml)	Estimated concentration (μg/ml)	
40	38.57 ± 0.26	35.77 ± 0.40	37.24 ± 1.46	3.76
80	78.61 ± 1.20	77.39 ± 2.00	77.98 ± 2.40	0.79
120	125.41 ± 2.32	123.26 ± 1.40	118.44 ± 3.60	3.0
240	239.06 ± 0.38	245.93 ± 3.42	241.91 ± 0.74	1.42

(Values are mean ± SD)

4.1.5. Estimation of matrix effects in milk system for the quantification of BCM 7

To observe the effect of complex matrices on analysis of BCM 7 in milk system, matrix effect was calculated by comparing absorbance of BCM 7 in pure solvents to the milk system. The complex matrix of milk has significant effect on BCM 7. Milk spiked BCM 7 showed 35-45% lower response as compared to its aqueous solution (Table 4.3).

Nguyen *et al*., (2014) used matrix based calibration standards for quantification of BCM 7 in yogurt and reported 26-40 % lower signal intensity in yoghurt matrix standards compared to solvent based calibration standards and suggested additional cleaning of samples by solid phase extraction column to minimize the matrix effect. The RP-HPLC method successfully standardized in milk matrix system, able to detect BCM 7 in milk and their digestive extracts. Muehlenkamp and Warthensen (1996) and Jarmolowska *et al*. (2007) used the RP-HPLC method for the analysis of β-casomorphins in milk and milk products.

Table 4.3. Estimation of matrix effects in milk system for the quantification of BCM 7 in milk system

Concentration of BCM 7 (μg/ml)	Area of BCM 7in pure solvent	Area of BCM 7 spiked in milk	% Matrix Effect
15	451003	246802	45.28
30	811286	521027	35.78
45	1011286	674555	33.30
60	1322549	919828	30.45

The RP-HPLC method successfully standardized in milk Matrix, able to detect BCM 7 in milk system. Muehlenkamp and Warthensen (1996) and Jarmolowska *et al*., (2007) also applied RP-HPLC based method for the analysis of β-casomorphins in milk and milk products.

4.2. Effect of thermal processing on release of BCM 7 in heat processedmilk prepared from A1 and A2 raw milk

4.2.1. Proximate composition of A1 and A2 raw milk

Proximate analysis of A1 and A2 raw milk sample was carried out as depicted in Table 4.4. There was no significant difference in the chemical composition of both type of milk ($p>0.05$). Our findings were in accordance with Mukherjee *et al*. (2017) for Karan Fries cow milk though the authors nither mention the genotype of the animals and nor variant of β-CN in milk. These findings suggested that genetic variation of β-CN did not have any influence on basic composition of the milk.

Table 4.4. Proximate composition of A1 and A2 raw milk

Parameter	A1 raw milk	A2 raw milk
pH	6.43 ± 0.20	6.40 ± 0.10
Somatic cell count (10^3 cell/ml)	86.42 ± 14.55	83.00 ± 17.14
Fat (%)	3.40 ± 0.16	3.45 ± 0.19
Protein (%)	3.05 ± 0.15	3.05 ± 0.11
Casein (%)	2.30 ± 0.07	2.28 ± 0.08
Mineral (%)	0.70 ± 0.015	0.72 ± 0.017

(Values are mean ± SD, n=12)

4.2.2. Effect of simulated *in-vitro* digestion on proteolysis of A1 and A2 raw and processed milk

4.2.2.1. Degree of Hydrolysis during simulated digestion of A1 and A2 raw and processed milk

In order to assess the relation between extent of proteolysis and occurrence of BCM 7, degree of hydrolysis was calculated in terms of mmol of leucine release per gram of protein using standard calibration curve of L-leucine shown in Fig. 3.9. It was found that there was no hydrolysis in oral phase because saliva did not contain any proteolytic enzyme as depicted in Fig. 4.1.

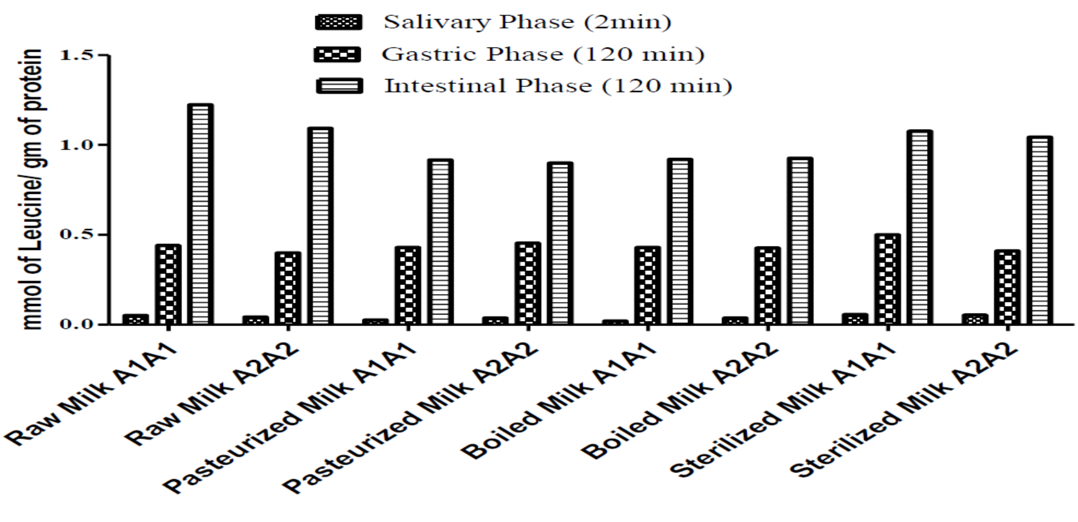


Fig 4.1. Effect of simulated *in-vitro* digestion on proteolysis of A1 and A2 raw and processed milk

In gastric phase around 40-50 % proteolysis occur in 2 h during gastric digestion. The maximum digestion of milk protein took place after intestinal digestion. There was no significant difference between digestive patterns of both variants ($P>0.05$). The highest degree of hydrolysis was observed during intestinal digestion of raw milk. The extent of proteolysis was least in intestinal extract of boiled milk followed by pasteurized milk (Fig. 4.1). However sterilized milk showed more proteolysis compare to boiled milk.

4.2.4.2. RP-HPLC chromatogram of water soluble gastrointestinal extracts of raw and processed milk

RP-HPLC profile of water soluble extracts prepared from *in-vitro* gastrointestinal digestive extracts of raw and processed milk was carried out to observe the extent of proteolysis by measuring the number of peaks identified in chromatogram. RP-HPLC chromatogram showed highest proteolysis in raw milk followed by sterilized milk (Fig. 4.2). Similar trend was observed by evaluating degree of hydrolysis as an index of proteolysis using TNBS assay. The higher degree of hydrolysis in sterilized milk may be due to thermal degradation of proteins during sterilization. The extent of hydrolysis of proteins especially casein is enhanced by dephophorylation, which is favored by the thermal treatment (Cattaneo *et al*., 2017). Severe heating of sodium caseinate at 130^0C showed thermal proteolysis. The majority of fragments identified during thermal degradation were from N or C – terminal region of casein and predominant cleavage sites were Pro, Ser, Asp, Asn amino acid residue containing peptide bonds (McGrath *et al*., 2016).

Similarly *in-vivo* digestion of pasteurized and UHT milk in human adults showed lesser proteolysis in case of pasteurized milk i.e. only 10% peptides were generated from β-CN in pasteurized milk compare to UHT milk showing 30% peptides released (Aalaei 2021).

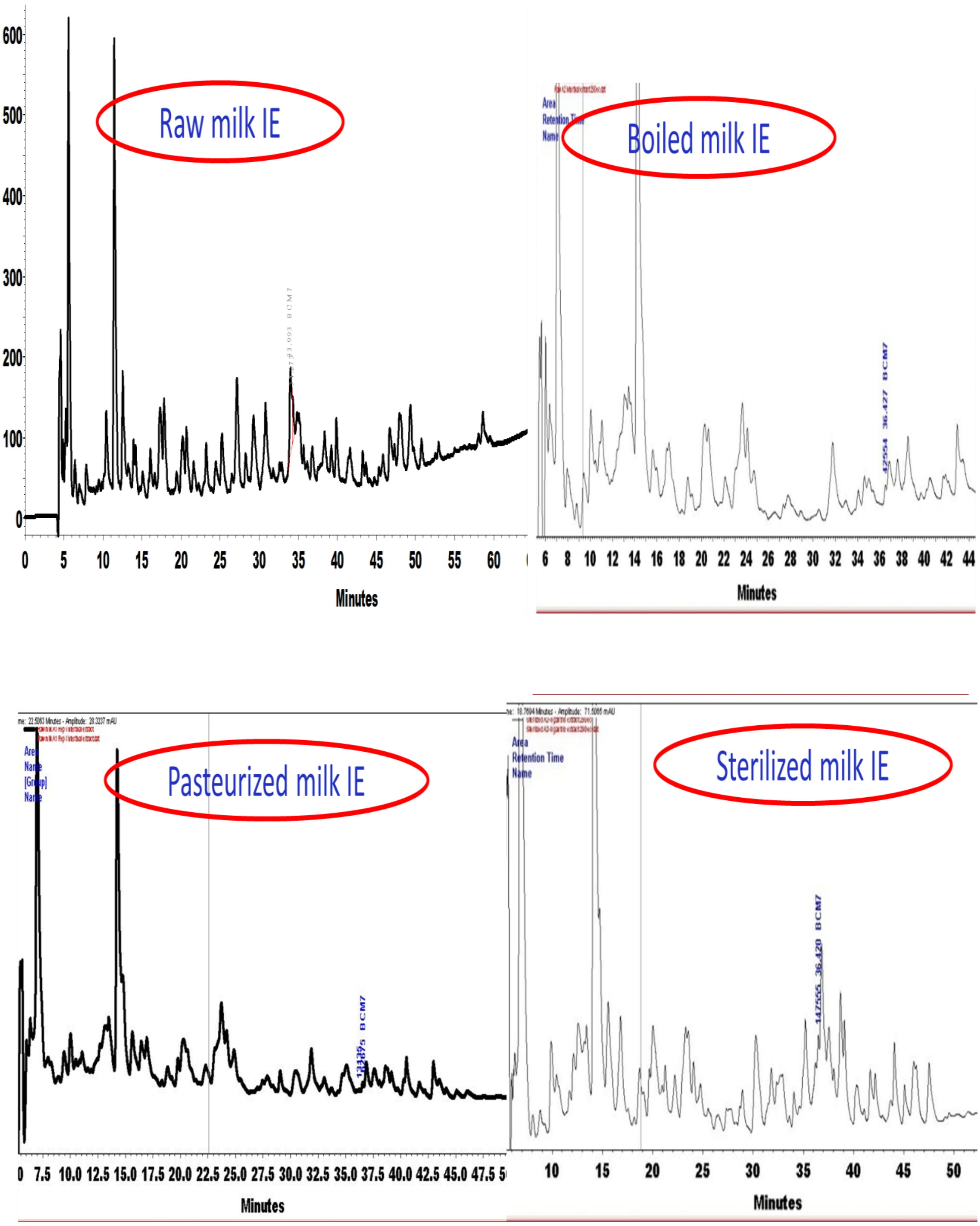


Fig 4.2 RP-HPLC chromatogram of water soluble extracts of A1 raw milk after simulated *in-vitro* gastrointestinal digestion (number of peaks showing extent of proteolysis).

4.2.5. Effect of heat processing on release of BCM 7 in thermally processedmilk prepared from A1 and A2 milk measured by RP-HPLC method

Thermal treatment is most common processing techniques in dairy industry to ensure safety and self life of dairy product. Different proteins behave differently on heat processing as it affects the native structural of proteins. To observe the effect of thermal processing on formation or degradation of BCM 7, three common treatments such as pasteurization, boiling and sterilization were used for the processing of milk. A2 and A1 Raw milk procured from A2A2 and A1A1 genotype KF cow respectively. A2 milkwas used as control.

BCM 7 was not detected in A1 and A2 raw and processed milk from A1A1 and A2A2 genotype cows, respectively (Table 4.5). These findings provide the evidence that release of BCM 7 requires a set of enzymes. Jinsmaa and Yoshikawa (1999) and Considine (1999) explain the release of BCM 7 by the action of series of proteolytic enzymes such as pepsin, trypsin, and elastase. Pepsin cleaves peptide bond between Leu_{58}-Val_{59} and then Leucine amino peptidase (LAP), removes Val from N-terminus of β-CN fragments to release BCM 7. De Noni and Cattaneo *et al*. (2010) also did not find BCM 7 in raw milk. Some studies reported contradictory results i.e. Cieślińska *et al*., (2007, 2012), Fiedorowicz *et al*. (2014) observed BCM 7 in raw milk from A1A1, A1A2as well as A2A2 genotypes cows. However highest concentration of BCM 7 was identified in milk from A1A1 genotype cow. In all above research authors did not mention about the status of raw milk in terms of somatic cell count, microbial load and indigenous proteases concentration. As raw milk contain fair amount of micro floura, somatic cells as well as endogenous proteolytic enzymes. Above mentioned factor may be responsible for the presence of BCM 7 in raw milk (EFSA 2009). Dallas *et al*., (2014) also reported the presence of BCM 7 and pro BCMs in raw milk, but authors did not specify the genetic variant of β-casein in milk. Recently Nguyen *et al*. (2019) identified BCM 7 in raw milk of Holstein Friesian breed. This bovine breed majorly contains A1 type of β-CN variant. In our study, absence of BCM 7 in pasteurized, boiled and sterilized milk of both genotypes showed that heating alone cannot generate BCM 7(Table 4.5). De Noni and Cattaneo (2010) also reported the absence of BCM 7 in heat processed milk.

Table 4.5. Quantification of BCM 7 in heat processed milk and in their simulated *in-vitro* digestive extracts prepared from A1 and A1 milk

	Raw Milk		Pasteurized Milk		Boiled Milk		Sterilized Milk	
	A1	**A2**	**A1**	**A2**	**A1**	**A2**	**A1**	**A2**
Control	nd	Nd	nd	nd	nd	nd	nd	nd
Salivary Extract	nd	Nd	nd	nd	nd	nd	nd	nd
Gastric Extract	nd	Nd	nd	nd	nd	nd	nd	nd
Intestinal Extract (ng/ml)	1399.32 ± 151.21	97.44 ± 21.04	699.11 ± 194.01	46.82 ± 14.82	372.83 ± 142.72	22.54 ± 12.02	215.95 ± 21.02	14.82 ± 5.17
Range (ng/ml)	900.99 - 1612.76	74.00 - 146.75	340.06 - 1132.16	46.10 - 79.06	192.05 - 792.06	14.56 - 58.30	180.86 - 312.86	13.06 - 25.32

(n=12, values are mean ± SD)

As expected simulated *in- vitro* digestive study of raw milk and processed milk showed that 2 min oral digestion did not have any effect on milk proteins (Fig. 4.3) and no BCM 7 observed in salivary extract of all samples (Table 4.5). As there is no proteolytic enzyme in saliva hence no hydrolysis during oral phase (Benedé *et al*., 2014).

BCM 7 was not observed in the gastric extracts of raw, pasteurized, boiled and sterilized milk as depicted in Table 4.5. Nguyen *et al*. (2020) also reported that no pro BCMs or BCM 7 released after 2 h of gastric digestion of cow milk. Recently Lambers *etal*. (2021) studied the processing effect on release of BCM 7 during different digestion stages and reported that no pro BCMs and BCM 7 were released in raw and heat processed milk during gastric digestion. Similar findings were reported by Asledottir *et al*. (2017) and De Noni (2008). Even gastric digestion of purified β-CN fraction unable to generate BCM 7 (Ul Haq *et al*., 2015). Digestion of proteins molecules starts in the stomach where pepsin is only enzyme present in gastric juice acts on specific site in protein chain. Thereby only partial hydrolysis takes place during gastric digestion. Jinsmaa and Yoshikawa (1999) and Considine (1999) explains the release of BCM 7 that require a set of enzymes such as pepsin, trypsin, elastase. Firstly pepsin cleaves the peptide bond between Leu58-Val59 and pancreatic enzymes such as trypsin and chymotrypsin break down peptide bonds at basic and aromatic amino acid residues respectively to generate of large molecular weight peptides. Then Val is releases by leucine amino peptidase (LAP), from N-terminus of β-CN fragment whereas pancreatic elastase releases amino acid residue from C-terminus by cleavage of Ile66-His67 due to its broad specificity towards β- CN molecule (Jarmolowska *et al*., 1999). The other cleavage site of elastase in β-CN are Ile26-Asn 27, Gln40-Thr41, Ile49-His50, Phe52-Ala53, Gln56-Ser57, Leu58-Val59, Asn68-Ser69, Val82-Val83, Val95-Ser96, Ser96-Lys97, Lys97-Val98, Ala101-Met102,Glu108-Met109 (Considine *et al*., 1999). Therefore generation of BCM 7 is outcome of complex action of number of peptidases. Thereby pepsin alone is unable to generate BCM 7 (Ul Haq *et al*., 2015; Jinsmaa and Yoshikawa 1999). A study was conducted by Benedé *et al*. (2014) to assess the digestion pattern of β-CN by porcine pepsin enzyme using simulated gastric and human gastric fluid. They reported that a large numbers of peptides generated during gastric digestion. Nearly 152 peptides were released with varying chain length between 3 and 21 amino acid residues and showing sequence coverage of 67.0% and 80.4%. In spite of release of so many small chain peptides, release of BCM 7 was not identified by the authors. Recently Nguyen *et al*., (2020) also reported that gastric digestion did not release any BCM 7 or pro BCMs in commercial yoghurt. Authors also identified the cleavage sites of β-CN during digestion and mentioned that most of peptides released were from the 91-111 region of the molecule. Contradictory to above findings some studies identified BCM 7 in gastric extracts of milk and milk products (Cieślińska *et al*., 2012 De Noni and Cattaneo 2010; De Noni *et al*, 2015).

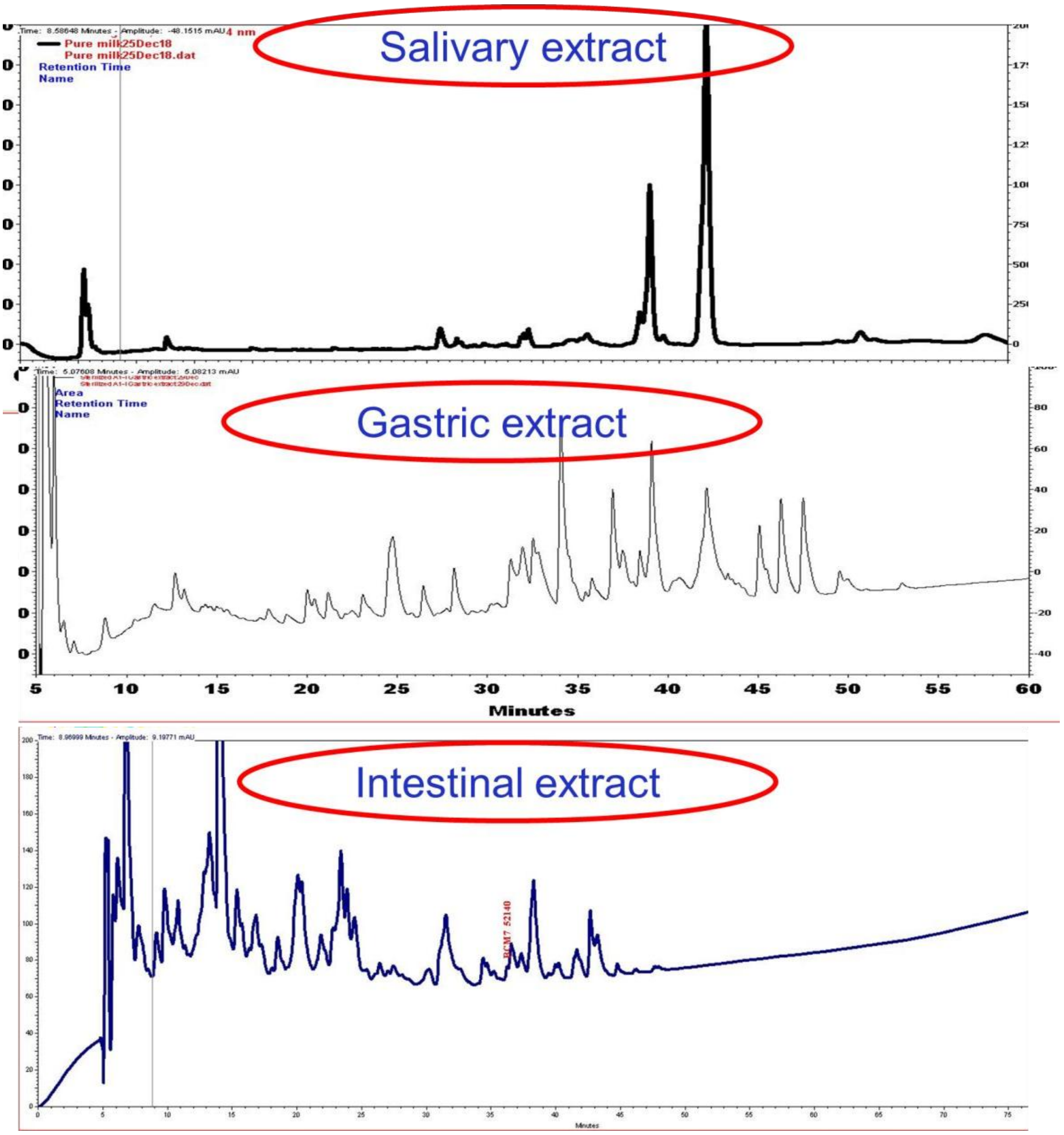


Fig 4.3. Typical RP-HPLC chromatogram of raw milk at different stages of simulated *in-vitro* digestion showing the release of BCM 7 during gastrointestinal digestion (red marking)

Release of BCM 7 was observed in all samples of A1 and A2 raw cow milk after simulated *in-vitro* gastrointestinal digestion (Table 4.5). Concentration of BCM 7 was 1399.32 ± 151.21 ng/ml in A1 raw milk from A1A1 genotype cow significantly higher compared to A2 raw milk (A2A2 genotype cow) (97.44 ± 21.04 ng/ml) ($p<0.05$). Gastrointestinal extracts of all processed A1 cow milk samples exhibits about 14-15 times higher concentration of BCM 7 compares to A2 cow milk. Results obtained in the study were in correlation with the previous studies reported in literature. Miguel *et al*. (2017) also identified significantly higher concentration of BCM 7 from gastrointestinal digestive extracts of Holstein Friesian (A1A1 genotype) raw milk (2.11 ± 0.19 µg/100 ml) compare to Jersey (A2A2 genotype) milk (0.74 ± 0.008 µg/100 ml). Similarly Fiedorowicz *et al*. (2014) studied the presence of BCM 7 in milk and their enzymatic extracts of Holstein Friesian bovine breed and reported that milk containing A1 β-CN variant posses higher concentration of BCM 7 in milk as well as their enzymatic extracts compare to milk having other variants of β-CN. These authors also reported that concentration of BCM 7 was 13-36 times higher in the enzymatic hydrolysed milk compare to control milk. Similar findings were reported by Asledottir *et al*. (2017 and 2018) and Cieślińska *et al*. (2007) in raw milk. Findings of our study also suggested that higher concentration of BCM 7 in milk from A1A1 genotype cow may be related to specific amino acid residue at 67th position in β-CN sequence. It is well reported in literature that under normal digestive condition A2 β-CN releases negotiable or no BCM 7 (Jinsmaa and Yoshikawa 1999; Cieślińska at al., 2007; De Noni 2008; EFSA 2009; Miguel *et al*., 2017; Ul Haq *et al*., 2015). Apart from amino acid specificity, milk containing A1 β-CN variant posses better physiochemical properties such as better coagulation characteristics and higher surface hydrophobicity and hydrodymanic diameter compare to milk with other β-CN variants (Ketto *et al*., 2017; Poulsen *et al*., 2017). These physicochemical characteristics enhanced the digestibility of β-CN by gastrointestinal enzymes. Hence more probability of generation of short chain BCM 7 likes peptides. The pro BCMs and BCM 7 were most frequent proline rich peptides found in the intestinal extracts of healthy human after consuming of milk and milk products (Boutrou *et al*., 2013). Although the concentration of these peptide decreased with time, but BCM 7 continuously released within the human intestinal after taking casein diet. The results obtained in our study as shown in Table 4.5, supports the theory of milk containing A2 β-CN variant is more resistant to digestion which may lead to generation of less BCM 7 in milk from A2A2 genotype cow. Contradictory to our findings, recently Nguyen *et al*. (2021) reported that no BCM 7 was released after gastrointestinal digestion in reconstituted milk having β-CN A2 variant, but identified in the gastrointestinal extracts of milk consist of β-CN A1 (127.25 - 198.10 ng/ml) and β-CN A2/I variants (19.35 -24.50 ng/ml).

On comparing thermal processing treatments, gastrointestinal extracts of A1 raw milk from A1A1 genotype cows showed highest release of BCM 7 peptide (Table 4.5). It may be due to the combine action of gastrointestinal and native enzymes of raw milk. Proteinases such as, pepsin, cathepsin B and D, elastase or other minor proteases able to form number of peptides in raw milk (Kelly *et al*., 2006, Albenzio *et al*., 2009). Beside the action of native proteases, microbial proteases also promote proteolysis and responsible for release of BCM 7 from pro BCMs fragments (Nielsen 2002) as raw milk contains fair counts of microorganism.

Concentration of BCM 7 in gastrointestinal extract of milk within same genotype varies distinctly in the range of 74-146 ng/ml in A2 and 900-1612 ng/ml A1 milk from A2A2 and A1A1 genotype cows respectively (Table 4.5). It might be due to variation in stages of lactation of selected animals for the study, as they were not of same lactation age group. Concentration of indigenous enzymes varies throughout the lactation (Albenzio *et al*., 2009). Hence each milk sample has specific concentration of native enzymes and therefore specific enzymatic activity.

Presence of BCM 7 observed in gastrointestinal extracts of all pasteurized A1 and A2 cow milk depicted in Table 4.5. The content of BCM 7 in A2 pasteurized cow milk was in the range of 46.0 - 79.0 ng/ml significantly lower than A1 milk (340-1132 ng/ml) ($P<0.05$). About 50-52% reduction in release of BCM 7 was observed after LTLT pasteurization (63^0C for 30 min) as shown in Fig. 4.4. An inhibitory effect of heat treatment on generation of BCM 7 was independent to the types of genotypes.

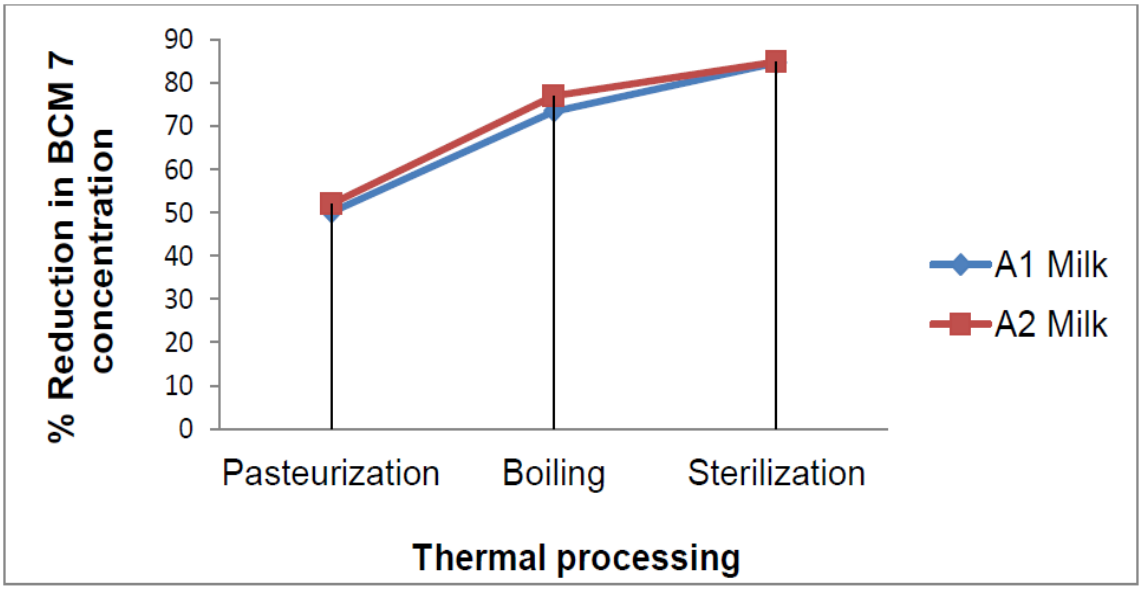


Fig 4.4. Effect of thermal processing on release of BCM 7 like peptides during *in- vitro* simulated gastrointestinal digestion of A1 and A2 milk

As pasteurization treatment causes inactivation of natural micro flora of milk resulting in lower proteolysis, thereby lower content of BCM 7 peptide. About 73.35-76.86 % reduction in release of BCM 7 was observed in intestinal digestive extracts after application of boiling treatment to the A2 and A1 milk (Fig. 4.4). The trend of reduced concentration was identical in both genotypes. Similar results were reported by Cieślińska *et al*., (2012) that heat treatments have inhibitory effect on the release of BCM 7 peptide. Severity of thermal processing increased in sterilized milk so further reduction in released of BCM 7 was observed (Fig. 4.4). The intestinal extracts of milk from both genotypes showed the lowest concentration of BCM 7 compare to boiling and pasteurization. When sterilization treatment was applied to milk samples about 84.79 and 84.56% reduction in the generation of BCM 7 observed from A2 and A1 sterilized milk during intestinal digestion.

Reduction of BCM 7 during thermal processing of milk may be explained by the well known fact that during heat processing, proteins undergo physicochemical changes such as glycosylation, dephosphorylation, aggregation and polymerization (Fox *et al*., 2015). These chemical changes may affect the protein digestibility, may increase or decrease the proteolysis in gastrointestinal digestion (Gilani *et al*., 2012; Kananen *et al*., 2000). These findings suggests that heating may be affect the release pro BCMs and BCM 7 peptide (Barbe *et al*., 2014). Formation of thermally induced aggregates between caseins and whey protein and other structural changes may influences heat stability of proteins and affect enzymatic activity of digestive enzymes (Pinto *et al*., 2014; Deglair *et al*., 2019). Some studies reported that formation of casein-whey protein aggregates via disulphide bonds can impaired casein digestion (Barbe *et al*., 2014; Sanchez-Rivera *et al*., 2015; Aalaei *et al*., 2021).

At high temperature extensive glycation causes conformational changes in protein structure (Corzo-Martinez *et al*., 2007), which may leads to unavailability of specific site of β-CN to hydrolysis during gastrointestinal digestion. Thereby release of pro BCMs and BCM 7 peptide from milk and milk products may reduce during gastrointestinal digestion (Barbe *et al*., 2014).

Another reason for reduced concentration of BCM 7 especially in boiled and sterilized milk may be heat induced proteolysis (Hustinx *et al*., 1997; Ebner *et al*., 2016) leads to the further breakdown of pro BCMs and BCM 7 peptides. From our findings it was observed that degree of hydrolysis was highest in sterilized milk among the heat processed milk (Fig 4.2), which showed that highest proteolysis took place during gastrointestinal digestion in sterilized milk of both genotypes. Gaucheron *et al*. (1999) also reported the release of low molecular weight casein derived peptides during sterilization. Some studies revealed that sterilization has significant effect on peptide profile of milk proteins. Especially β-CN molecule undergoes more proteolysis and generates more peptides than pasteurized milk after digestion (Aalaei *et al*., 2021). Similar finding were reported in another study that proteomic profile of UHT treated milk showed about 30% peptides generated from β-casein and 10% in pasteurized milk after gastrointestinal digestion (Ebner *et al*., 2016). Although their peptide profile were very similar to each other before digestion. Heat processing of sodium caseinate at 140^0C showed extensive proteolysis due to physicochemical changes such as dephosphorylation, intermolecular cross-linking etc. Majority of peptide fragments released by the breakdown of bonds involving Pro, Ser, Asp, Asn amino acid containing peptide bonds, and generated peptides were evenly distributed throughout the β-CN sequence (Hustinx *et al*., 1997; McGrath *et al*., 2016). These researchers observed that major cleavage sites identified in β-casein, were Val_8 -Pro_9, Asn_{27}-Lys_{28}, Asp_{43}-Glu_{44}, Gln_{54}-Thr_{55}, Val_{59}-Tyr_{60}, Pro_{63}-Gly_{64}, Asn_{68}-Ser_{69}, Pro_{76}-Leu_{77}, Gln_{79}-Thr_{80}, Thr_{80}-Pro_{81}, Val_{95}-Ser_{96}, Ser_{96}-Lys_{97} and His_{148}-Gln_{149}. Out of these two cleavage Val_{59}-Tyr_{60} and Asn_{68}-Ser_{69} may be responsible for the generation of BCM 7 or BCM 7 like peptides. Heat induced specific cleavage especially between 50-81 position of β-CN may generate number of peptides which further hydrolyzed by gastrointestinal enzymes leading to production of low molecular weight peptide i.e tri or di peptides.

Release of BCM 7 during *in-vitro* simulated gastrointestinal digestion decreased with enhanced severity of thermal processing (Fig. 4.4). The intestinal extracts of raw milk contains highest amount of the BCM 7 followed by pasteurized, boiled milk and sterilized milk ($P<0.05$). Similar trends were reported by Cattaneo *et al*. (2017). Recently Lambers et el. (2021) also studied the effect of heating temperature on formation of BCM 7 during simulated gastrointestinal digestion of A2 and A1 milk and reported that release of BCM 7 inhibited by typical pasteurization and UHT treatment in milk from both genotype in similar manner. Contrary to above findings Noni and Cattaneo (2010) reported that heat processing did not have any impact on BCM 7.

4.2.6. Effect of heat processing on release of BCM 7 during gastrointestinal digestion of thermally processed A1 and A2 milk measured by ELISA assay

Application of ELISA based assay for the quantification of BCM 7 in milk and milk products were used by a number of researcher, being a highly sensitive and easy to perform (Sienliewicz-Szłapka *et al*., 2009; Cieślińska *et al*., 2012; Nguyen *et al*., 2018). There by to validate the results of RP-HPLC methods, gastrointestinal digestive extracts of raw and processed milk were analyzed by ELISA assay. Identification and quantification of BCM 7 by ELISA assay also showed similar trend of occurrence of BCM 7 in all samples as identified by RP-HPLC method as depicted in Fig 4.5. Although the concentration of BCM 7 obtained by ELISA assay were significantly lower ($P<0.05$) than the concentration estimated by RP-HPLC method.

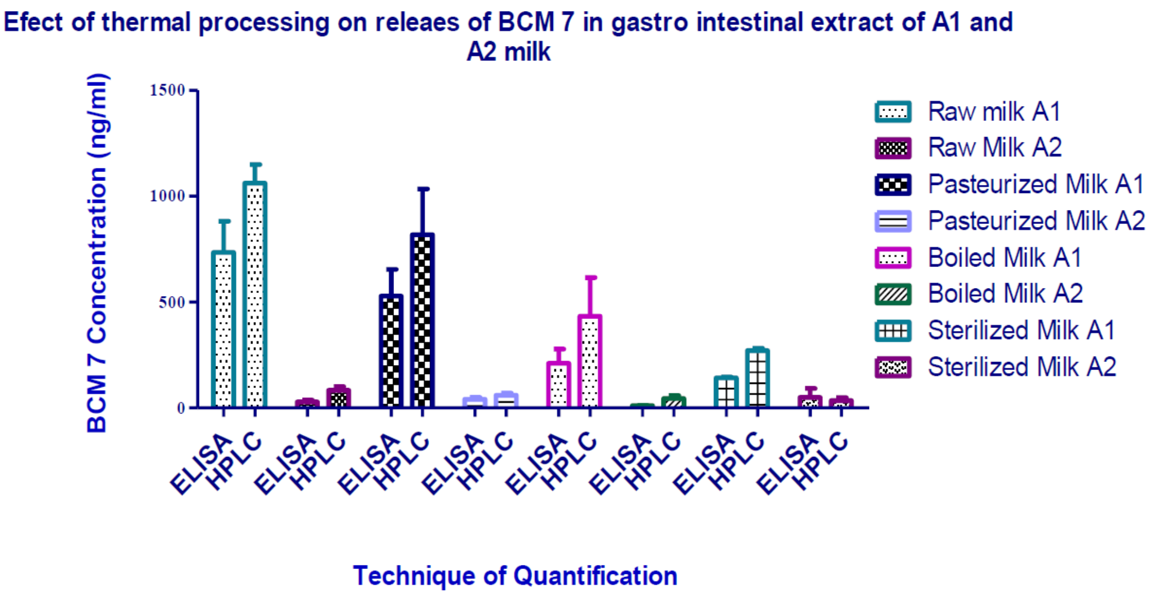


Fig. 4.5. Quantification of BCM 7 using RP-HPLC and ELISA in the gastrointestinal extracts of raw and heat processed A1 and A2 milk

As severity of heating enhanced concentrations of BCM 7 decreased in gastrointestinal extracts of processed milk i.e. sterilized milk showed lowest release of BCM 7 during simulated gastrointestinal digestion. Similar trends were also reported by Cattaneo *et al*. (2017) and Lambers et el. (2021) that thermal processing such as pasteurization and UHT treatment has inhibitory effect on release of BCM 7 during simulated gastrointestinal digestion of milk from A2A2 and A1A1 genotype cattle. ELISA techniquebeing more sensitive and selective provides more accurate and precise result compare to RP-HPLC. Difference in concentration in both methods may be due to overestimation of BCM 7 by RP-HPLC method. A number of peptides analogous in physicochemical characteristics may co-elute with BCM 7 in HPLC method and give rise to higher optical density so enhanced signal intensity. It is evident from section 4.2.7 of this study, LCMS results showed that a number of BCM 7 like peptides generated during gastrointestinal digestion of both types of milk. It is very difficult to integrate single peak in RP-HPLC chromatogram. Other reason of underestimation of BCM 7 concentration in ELISA assay may be maillard reaction between amino acid and lactose may modify the conformation of BCM 7 which may affect the binding affinity of modified BCM 7 to specific antibody used in ELISA technique (Nguyen *et al*., 2018).

4.2.7. Peptide identification in simulated *in-vitro* intestinal digestive extract of A1 and A2 raw milk by mass spectroscopy

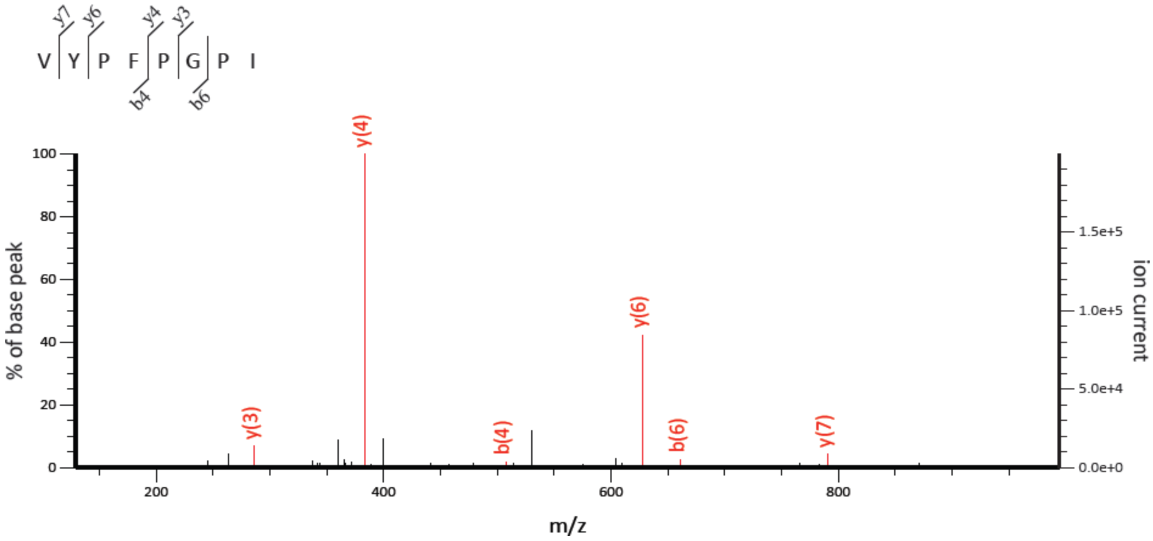
Lyophilized simulated crude *in–vitro* gastro-intestinal extract and specific HPLC fractions representative of BCM 7 from A1 and A2 raw milk were analyzed using ESI-QUAD-TOF. Peptides generated during simulated *in-vitro* gastrointestinal digestion were sequenced by SwissProt 2020_04x data base for bovine milk proteins. The enzyme used as "None" for searching the database. Approximately 144 peptides sequences of β-casein were identified in gastrointestinal digestive extracts of raw milk. Identified peptides were showing about 83% sequence coverage of β-CN molecules. However Boutrou et al. (2013) identified about 218 β-CN derived peptides with 61.2 % sequence coverage in human jejunal effluent of dairy products during digestion. Around 22 β-casein derived peptides were identified in the RP-HPLC fraction (±1 min) representing BCM 7 of intestinal extracts of A2 and A1 Milk (Table 4.6 and 4.7).

Most of peptides generated during digestion were completely or partially matching with the sequences previously identified from gastro-duodenal digestion of β-CN using *in- vitro* and *in-vivo* digestive conditions (Boutrou *et al*., 2013 and Benede *et al*., 2014). In present study we were able to identified number of peptides with varying length generated from β-CN fragment (54-72) during gastrointestinal digestion of milk from both genotype cows. A umber of BCM 7 like peptides were released from β-CN molecule as depicted in Table 4.6 after gastrointestinal digestion of A2 raw milk from A2A2 genotype cows, such as β (59-68), β (58-68), β (57-68), β (56-68), β (55-68) (MS spectra were shown in Fig. 4.6 a to g) . These peptides contain more number of proline residues and indicate that proline rich peptides are resistant to gastrointestinal digestion(Nano *et al*., 2020)

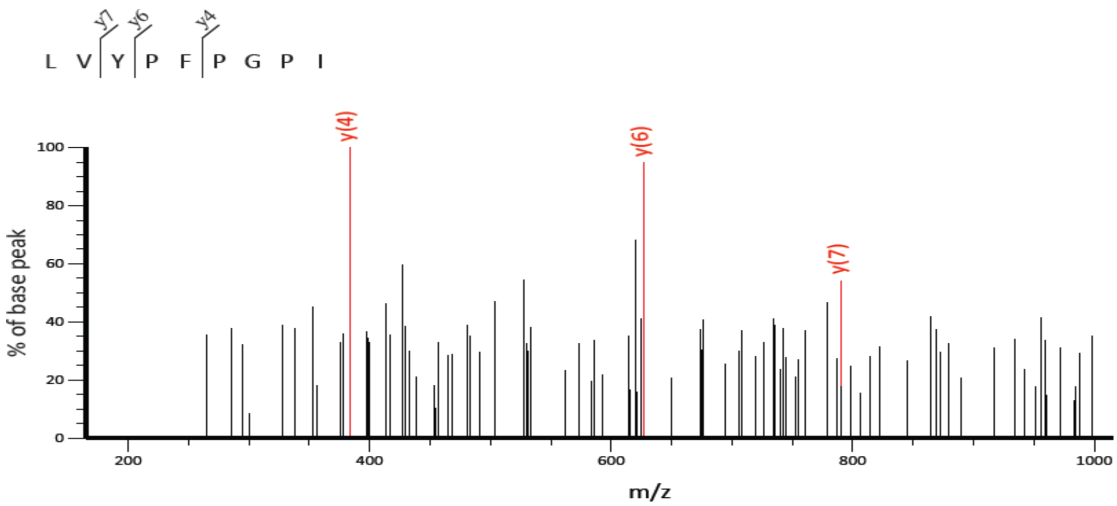
Table 4.6. β-Casein derived peptides in specific RP-HPLC fraction generated during *in-vitro* simulated gastrointestinal digestion of A2 raw milk

Peptide	Sequence (β-Casein)	Mass (Observed)	Bioactivity (sited in literature)	Peptides reported in intestinal extracts of milk(Reference)
VPPFL	84-88	572.3384	-	-
VLPVPQ	170-175	652.3981	-	Benede *et al*., (2014)
HLPLPL	34-39	689.4297	Antihypertensive ACE-inhibitory Anti-amnestic	Hernandez-Ledesma *et al*.,(2007) Ul Haq *et al*., (2015)Asledottir *et al*., (2017) Deglaire *et al*.,(2019)
GPFPIIV	203-209	742.4403	ACE-inhibitory	Hernandez-Ledesma *et al*.,(2007)
PVVVPPF	81-87	754.4415		
VLGPVRGP	197-204	397.7455	ACE-inhibitory	Tu *et al*., (2020)
AVPYPQR	177-183	415.7283	Antihypertensive	Hernandez-Ledesma *et al*.,(2007) Tu *et al*., (2020)
PVVVPPFL	81-88	867.5265		
VYPFPGPI	59-66	889.4748	-	Jinsmaa and Yoshikawa(1999) Tu *et al*., (2020)
LVYPFPGPI	58-66	1002.5605	-	Benede *et al*., (2014)Tu *et al*., (2020)
FLLYQEPV	190-197	1008.5296	-	-
LTLTDVENL	125-133	1017.5381	-	-
PVEPFTESQ	115-123	1033.4668	-	Benede *et al*., (2014)
NVPGEIVESL	1-16	1056.5468	ACE-inhibitory	Tu *et al*., (2020)
VYPFPGPIPN	59-68	1100.5734	ACE-inhibitoryAntioxidative	Jinsmaa and Yoshikawa(1999) Hernandez-Ledesma *et al*.,(2007) Asledottir *et al*., (2017)
LYQEPVLGPV	192-201	1114.6027	Immunomodulator ACE-inhibitory	Boutrou *et al*., (2013) Barbe *et al*., (2014) Asledottir *et al*., (2017)
VVPPFLQPEV	82-92	1124.6281	Antihypertensive	Benede *et al*., (2014)
LVYPFPGPIPN	58-68	1213.6543	-	Benede *et al*., (2014)
SLVYPFPGPIPN	57-68	1300.6947	Opioid	Schieber and Bruckner(2000)
SLSQSKVLPVPQ	164-175	1282.7472		-
QSLVYPFPGPIPN	56-68	1427.7449		
FQSEEQQQTEDE LQDKIHPF	38-52	826.0436	-	Ul Haq *et al*., (2015)
TQSLVYPFPGPIPN	55-68	1528.7926		

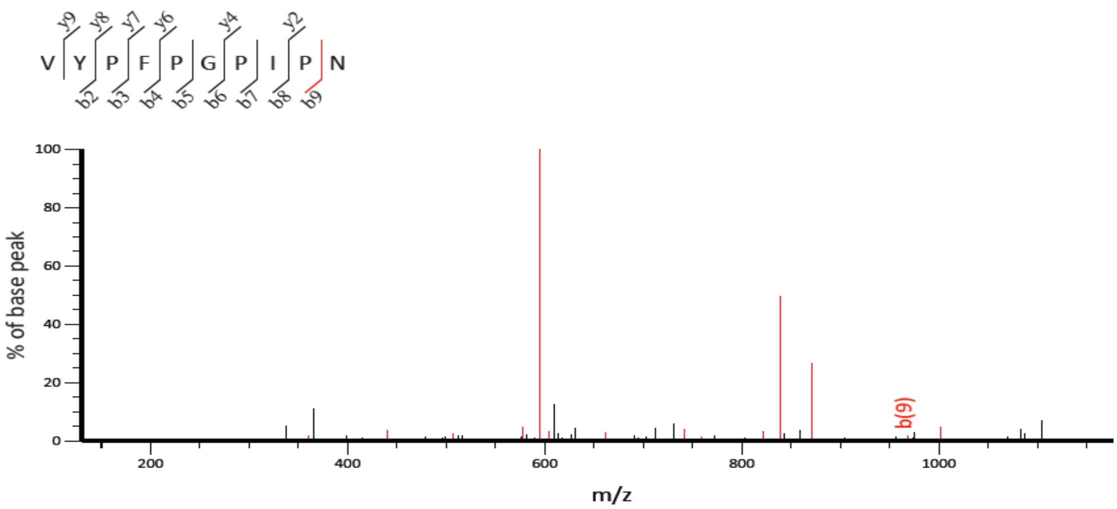
(Highlighted peptides are BCM 7 like peptides)



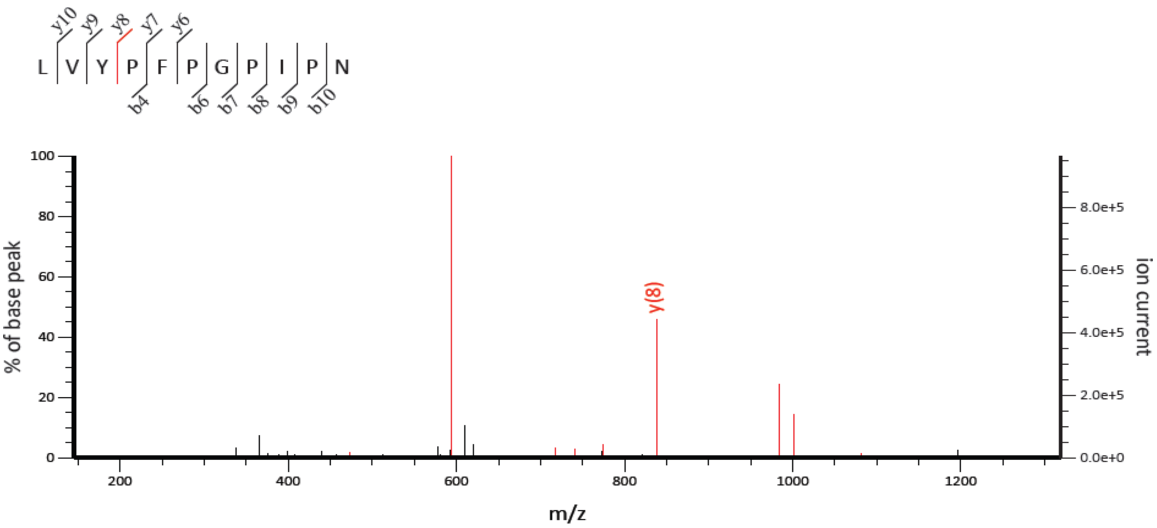
(a) BCM 7+1 peptide (VYPFGPI)



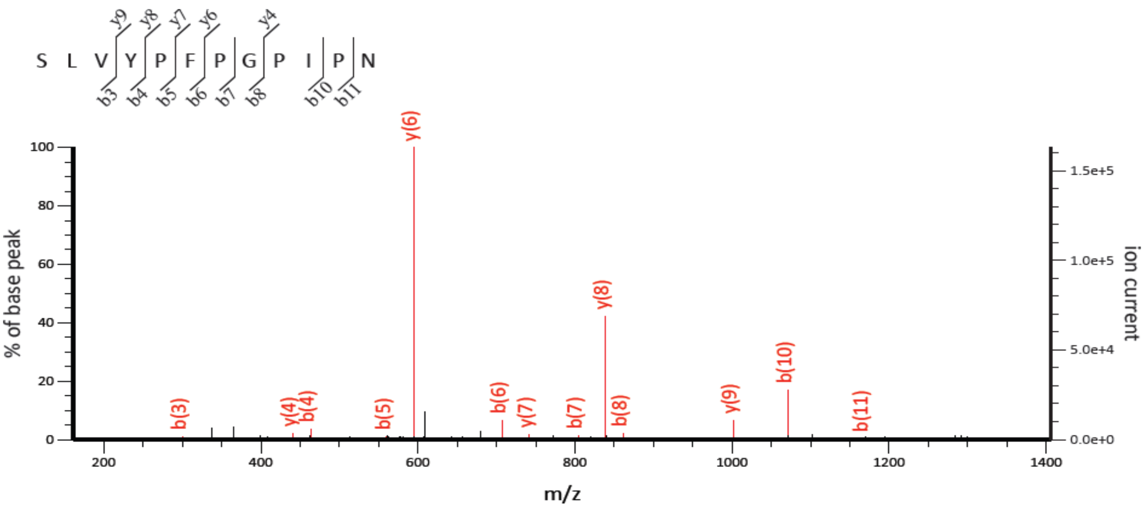
(b) BCM 7+2 peptide (LVYPFPGPI)



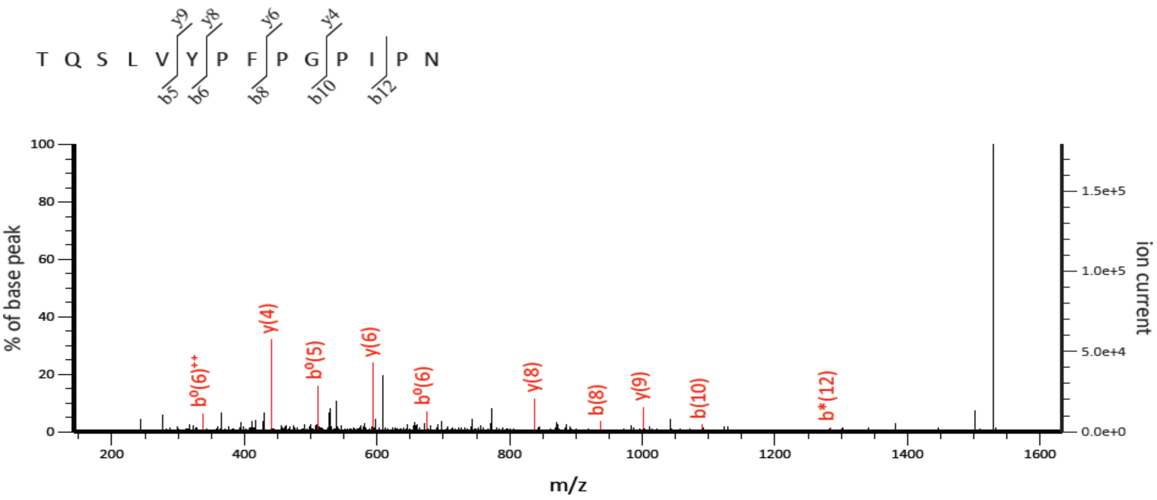
(c) Peptide VYPFPGPIPIN



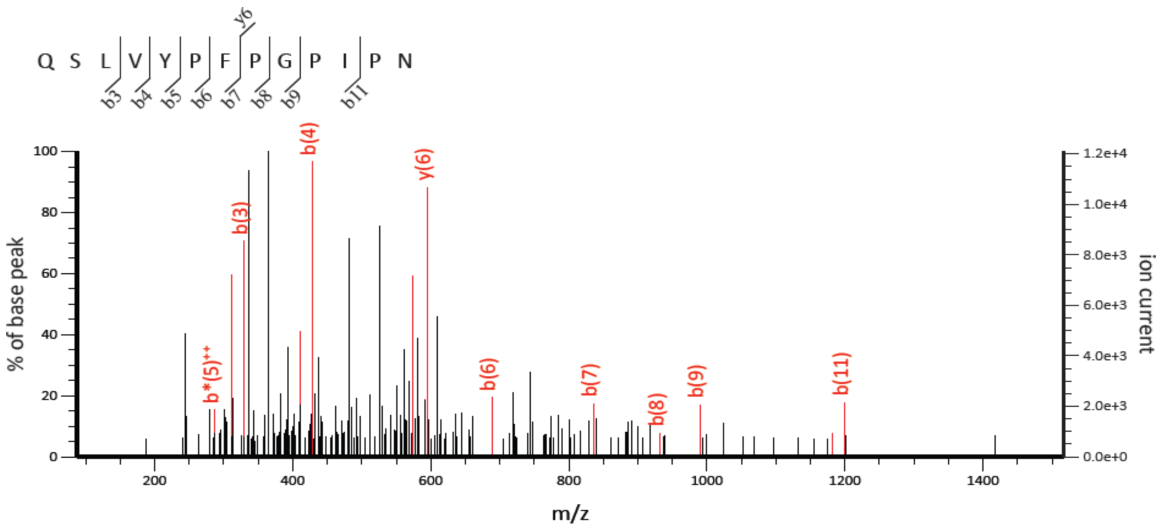
(d) Peptide (LVYPFPGPIPN)



(e) Peptide SLVYPEFPGIPN



(f) Peptide TQSLVYPFGPIPN



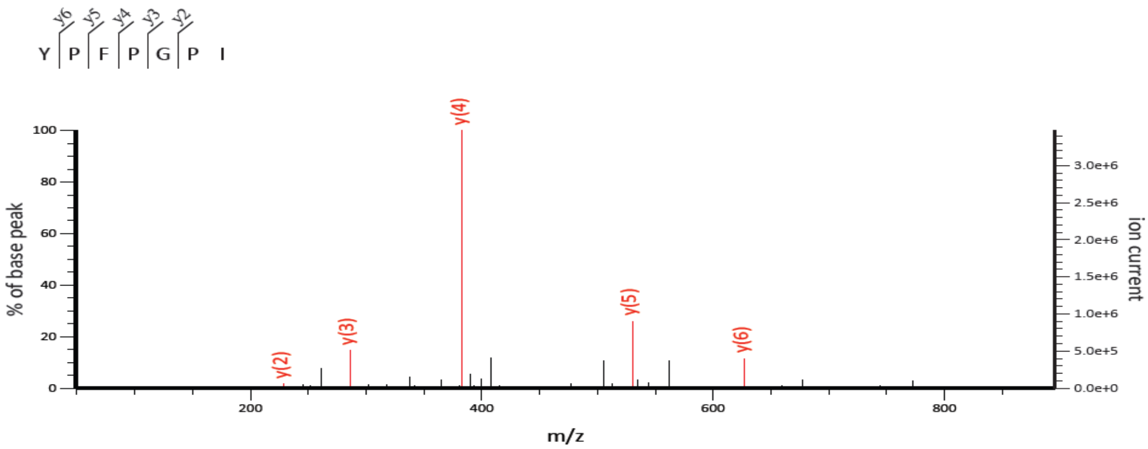
(g) Peptide QSLVYPFPGIPN

Fig. 4.6 (a-g). Ion fragmentation MS spectra of BCM 7 like peptides released during *in-vitro* simulated gastrointestinal digestion of A2 raw milk

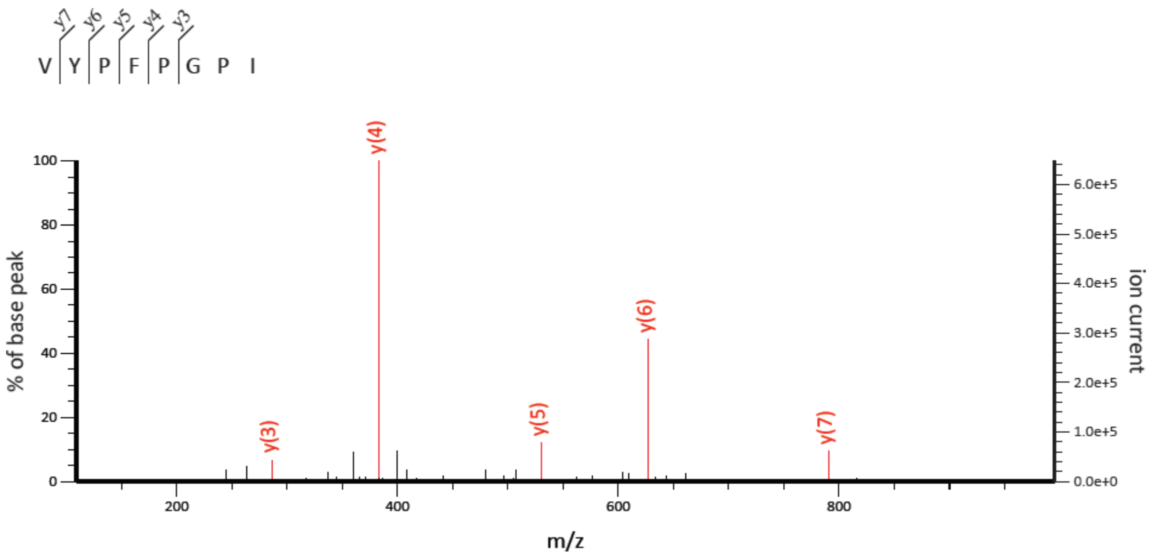
Table 4.7. β-Casein derived peptides in specific RP-HPLC fraction generated during *in-vitro* simulated gastrointestinal digestion of A1 raw milk

Peptide	Sequence (β-Casein)	Mass (Observed)	Bioactivity(sited in literature)	Peptides reported in intestinal extracts of milk (Reference)
AVPYPQ	177-182	674.3530	ACE-inhibitory	Hernandez-Ledesma *et al*.,(2007), Tu *et al*., (2020)
GPFPIIV	203-209	742.4501	ACE-inhibitory	Hernandez-Ledesma *et al*.,(2007) Tu *et al*., (2020)
EMPFPK	108-113	748.3741	-	Hernandez-Ledesma *et al*.,(2007)
VKEAMAPK	98-105	437.2454	-	Benede *et al*., (2014)
VLGPVRGP	197-204	397.7460	-	Benede *et al*., (2014)
VLPVPQK	170-176	780.4996	-	Benede *et al*., (2014)
GPVRGPFPI	199-208	939.5428	-	-
YPFPGPI	60-66	790.4148	Opioid	Jinsmaa and Yoshikawa (1999)Asledottir *et al*., (2017) Lambers *et al*., (2021) Nguyen *et al*., (2021)
VVPPFLQPEV	83-92	1124.6352		
VYPFPGPI	59-66	889.4818	Opioid	Jinsmaa and Yoshikawa (1999)Tu *et al*., (2020)
SLVYPFPGPI	57-66	1089.5995		Boutrou *et al*., (2013)Benede *et al*., (2014)
LLYQEPVLGPV	190-201	1227.7010	ACE-inhibitory Anticougulant	Ul Haq *et al*., (2015) Asledottir *et al*., (2017) Tu *et al*., (2020)
QSLVYPFPGPI	56-66	1217.6544		Boutrou *et al*., (2013)
SLTLTDVENL	124-133	1104.5822		-
VVPPFLQPEVM	83-93	1255.6753		
YPVEPFTESQSL	114-125	1396.6681		Boutrou *et al*., (2013)Ul Haq *et al*., (2015)
AQTQSLVYPFPGPI	53-66	759.4056		Tu *et al*., (2020)
QEPVLGPVR	194-202	635.8627		Ul Haq *et al*., (2015)
TESQSLTLTDVENL	120-132	1549.7617	-	
HKEMPFPKYPVEPF	106-119	873.4423	ACE-inhibitory	-
MHQPHQPLPPTV	144-155	691.3565	-	Ul Haq *et al*., (2015)
NIPPLTQTPVVVPPFLQPEV	73-82	1093.1218	ACE-inhibitory	Benede *et al*., (2014)
FQSEEQQQTEDELQDKIHPF	38-52	826.0436	-	Ul Haq *et al*., (2015)Tu *et al*., (2020)

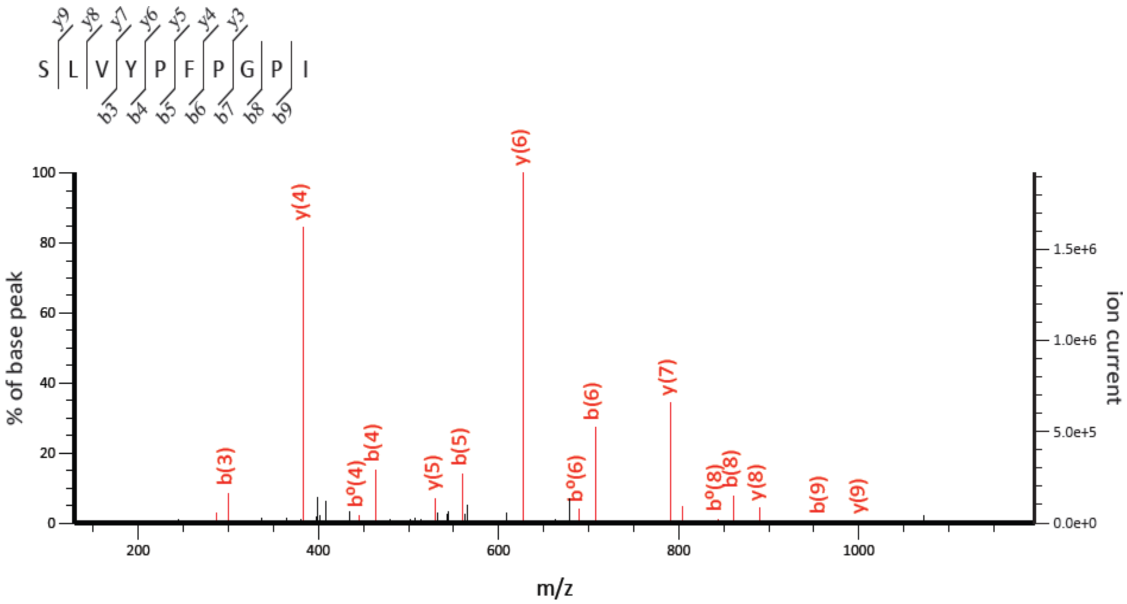
(Highlighted peptides are BCM 7 and BCM 7 like peptides)



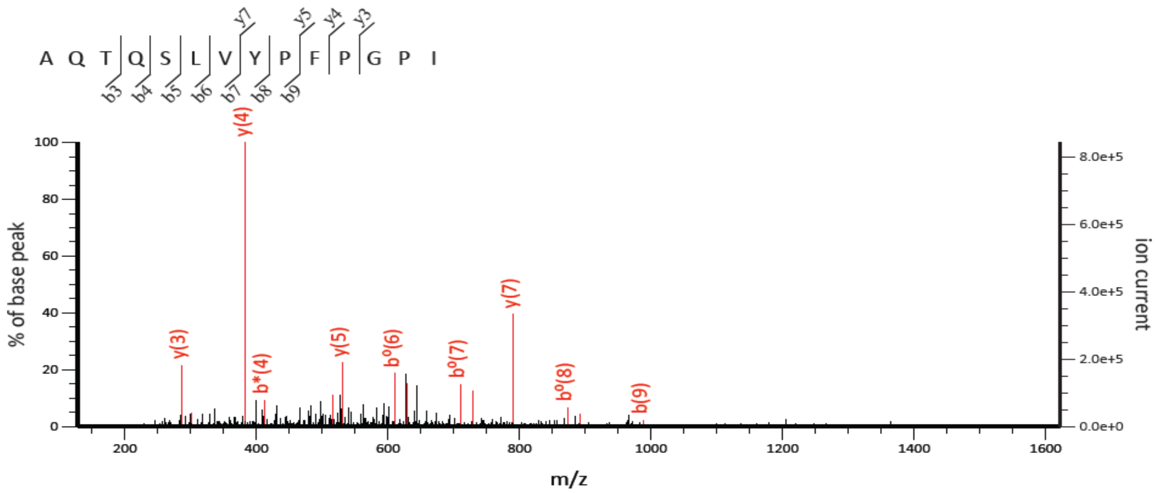
(a) BCM 7 peptide (YPFPGPI)



(b) BCM 7+1 peptide (VYPFPGPI)



(c) BCM 7+2 peptide (SLVYPFPGPI)



(d) Peptide AQTQSLVYPFPGPI

Figure 4.7 (a-d). Ion fragmentation MS spectra of BCM 7 and BCM 7 like peptides released during *in-vitro* simulated gastro intestinal digestion of A1 raw milk

Table 4.8. BCM 7 and BCM 7 like peptides generated during *in-vitro* simulated gastrointestinal digestion of raw bovine milk (Karan Fries) (Tharparkar X Holstein Friesian)

Peptide Sequence	Presence of peptide in A1 milk	Presence of peptide in A2 milk
^{60}YPFPGPI^{66}	YES	NO
^{59}VYPFPGPI^{66}	YES	YES
^{58}LVYPFPGPI^{66}	NO	YES
^{59}VYPFPGPIPN^{68}	NO	YES
^{58}LVYPFPGPIPN^{68}	NO	YES
^{57}SLVYPFPGPIPN^{68}	NO	YES
^{56}QSLVYPFPGPIPN^{68}	NO	YES
^{55}TQSLVYPFPGPIPN^{68}	NO	YES
^{53}AQTQSLVYPFPGPI^{66}	YES	NO
^{56}QSLVYPFPGPI^{66}	YES	NO

Results observed in our study were in agreement with the literature that Pro containing peptides considered resistant to gastro-intestinal digestion (Nano *et al*., 2000) as no peptides were identified showing cleavage at Pro-Gly (63-64), Gly-Pro (64-65). Asledottir *et al*. (2017) also reported the presence of β (60-68) and β (59-68) sequence in duodenal extract of milk from A2A2 genotype cattle. Benede *et al*. (2014) also identified β (59-68) during simulated duodenal digestion of β-CN, however author did not specify the genotype of the milking animal. Contradictory to these findings recently Lambers *et al*. (2021) reported the presence of β (60-68) in the gastrointestinal extracts of milk not only from A2 but also A1 milk. In addition to these peptides, we were able to identified number of BCM 7 like peptides in A2 milk from A2A2 genotype cow after intestinal digestion as shown in Table 4.6 and Fig 4.6, i. e. BCM 7 + 1 (^{59}**VYPFPGPI**^{66}**)** and BCM 7 + 2 (^{58}**LVYPFPGPI**^{66}). Although the frequency of occurrence of such peptides was less in milk from A2A2 genotype cow and our observations were in the line of previous study reported by Asledottir *et al*. (2017). We also observed a unique peptide ^{55}**TQSLVYPFPGPIPN**^{68} in gastrointestinal extracts of milk from A2A2 genotype cow (Table 4.6 and 4.8).

Benede *et al*. (2014) also reported ^{57}**VYPFPGPIPN**^{68}**,** ^{58}**LVYPFPGPIPN**^{68} and ^{58}**LVYPFPGPIPNSLPQ**^{72} after 30 min of simulated intestinal digestion of β-CN. Research carried out by Boutrou *et al*., (2013) showed that β-casomorphins such as β (59-66), β (59-68), and β (60-66) were the most frequent bioactive peptides generated during gastrointestinal digestion of casein fed diet in humans. In present study we did not able to identify BCM 7 in gastrointestinal extracts of A2 milk in spite of presence of number of pro BCMs and BCM 7 like peptides in the extracts. Surprisingly Lambers *et al*. (2021) identified β (60-65) and β (60-64) along with β (60-68) peptide in both genotypes after gastrointestinal digestion. Presence of BCM 6 and BCM 5 in digestive extracts indicated the probability of further hydrolysis of BCM 7 like peptides. Hence further hydrolysis of these peptides to BCM 7 peptide cannot be neglected in A2 milk also. Similarly Nguyen *et al*. (2020) and Nguyen *et al*. (2021) also reported that no BCM 7 released during gastrointestinal digestion of reconstituted cow milk and heat processed milk respectively. In our study release of BCM 7

(^{60}**YPFPGPI**^{66}) and BCM 7 like peptides such as ^{59}**VYPFPGPI**^{66}, ^{53}**AQTQSLVYPFPGPI**^{66} and ^{54}**QTQSLVYPFPGPI**^{66} were identified during gastro- intestinal digestion of A1 milk (Table 4.7 and 4.8).

It was also observed that relative abundance of these types of peptides were higher in gastrointestinal extracts of milk from A1A1 than A2A2 genotype cows. In case of intestinal extracts of milk from A2A2 genotype cows only two BCM 7 like peptides were identified (^{59}**VYPFPGPI**^{66} and ^{58}**LVYPFPGPI**^{66}) MS spectra were shown in Fig 4.6. Differences in the occurrence of these types of peptides in the digestive extracts of milk from both genotypes cows indicated that hydrolysis was prominently occurring between Ile$_{66}$-$_{67}$ Hiscompare to Ile$_{66}$-$_{67}$Pro peptide linkages. A number of studies reported that BCM 7 generated during gastro-intestinal digestion of milk containing A1 β-CN variant (Jinsmaa and Yoshikawa 1999; De Noni 2008; Cieślińska *et al*., 2012; Ul Haq *et al*., 2015; Asledottir *et al*., 2017; Nguyen *et al*., 2021; Lambers *et al*., 2021). Recently Tu *et al*., (2020) reported that β-CN derived peptides ^{59}**VYPFPGPI**^{66}**,** ^{58}**LVYPFPGPI**^{66} and ^{53}**AQTQSLVYPFPGPI**^{66} ^{57}**SLVYPFPGPIPN**^{68} released during *in-vitro* gastrointestinal digestion of casein have ability to cross caco-2 monolayer and may exhibit their physiological role in human beings. Ul Haq *et al*. (2015) also reported the presence of β(59-72) peptide in enzymatic hydrolysis of purified bovine casein extracted from A1A1 genotype bovine which may breakdown to BCM 7 on further hydrolysis. Lambers *et al*. (2021) also identified β (60-68), β (60-65) and β (60-64) in both genotypes after gastrointestinal digestion. Presence of β (60-65) and β (60-64) in digestive extracts indicates the probability of further hydrolysis of BCM 7 like peptides. Evidences are available in literature that BCM 7 like peptides can be hydrolysed to BCM 7 (Jinsmaa and Yoshikawa 1999; Jinsmaa Ul Haq *et al*., 2015; lambers *et al*., 2021).

The results obtained by RP-HPLC and ELISA showed the release of BCM 7 during gastrointestinal digestion of milk from both genotype cows, whereas LCMS sequencing analysis provide the evidence that BCM 7 generated in A1 milk during gastrointestinal digestion. Despite of occurrence of number of BCM 7 like peptides in gastrointestinal extracts, BCM 7 was not identified in A2 milk. From our study it can be inferred that BCM 7 does not release in A2 milk during gastrointestinal digestion, but presence of BCM 7 like peptides in the digestive extracts of A2 milk may contributes to the levels of BCM 7 during quantification by RP-HPLC and ELISA assay. It may be due to identical physicochemical characteristics of these peptides to BCM 7 peptide. It would be a better option to write BCM 7 like peptide instead of BCM 7 alone during reporting of results and presence of BCM 7 can be confirmed by peptide sequencing analysis using LCMS. Thus results obtained in our study supports the hypothesis that BCM 7 like peptides generate during gastrointestinal digestion of milk from both genotypes of cows , but cleavage is more specific to histidine at '67' position compare to proline at same position.

4.3. Studies on the release BCM 7 and BCM 7 like peptides during *in-vitro* simulated gastrointestinal digestion of *dahi* prepared from A1 and A2 cow milk

4.3.1. Preparation of *dahi* from A1 and A2 milk

Eight batches of *dahi* from each group were prepared according to standard procedure (given in 3.4.2.). Fresh milk from both genotypes were heated at 90^0C for 30 min, and cooled to 30 ± 2^0C. Subsequently, cultures prepared in pure genotype were added to their respective milk at 1.50% (w/v) level. The mixtures were put into sterilized glass containers and incubated at 30 ± 2^0C for 7-8 h. After incubation, *dahi* samples were stored at 5 ± 1^0C for one week. The samples were analyzed at 2 days intervals during storage.

4.3.2. Physicochemical characteristics of *dahi* prepared from A1 and A2 cow milk

Physicochemical and microbiological characteristics of fresh *dahi* were shown in table 4.9. There was no significant difference (P>0.05) between the parameters of *dahi* prepared from two different genotypes. pH, acidity, degree of proteolysis of both types of *dahi* were studied during storage at refrigerated temperature.

Table 4.9 Physicochemical analysis of fresh *dahi* prepared from A1 and A2 cow milk

Physicochemical Parameter	A1 *Dahi*	A2 *Dahi*
pH	5.11 ± 0.08	5.02 ± 0.12
Acidity (% lactic acid)	0.68 ± 0.02	0.70 ± 0.02
Fat (%)	3.41 ± 0.13	3.33 ± 0.18
Protein (%)	3.27 ± 0.12	3.42 ± 0.20
Total viable count(10^7cfu/ g)	14.98 ± 38	15.25 ± 50
Yeast and Mould countper g	nil	Nil
Coliform per g	nil	Nil

(values are mean ± SD where n=8)

4.3.3. Acidity and pH of *dahi* prepared from A1 and A2 cow milk

As expected all samples of *dahi* showed a decline in pH values and increased in acidity (Fig 4.8) with the storage period, which may be due to the growth of lactic acid bacteria during storage and production of lactic acid. Similar trend was explained by Deb and Seth (2014) that acidity increases with storage of *dahi*. Donkor *et al*., (2007a) also reported that during storage slight decline in pH observed in traditional and probiotic yoghurt. This effect is generally termed as post acidification.

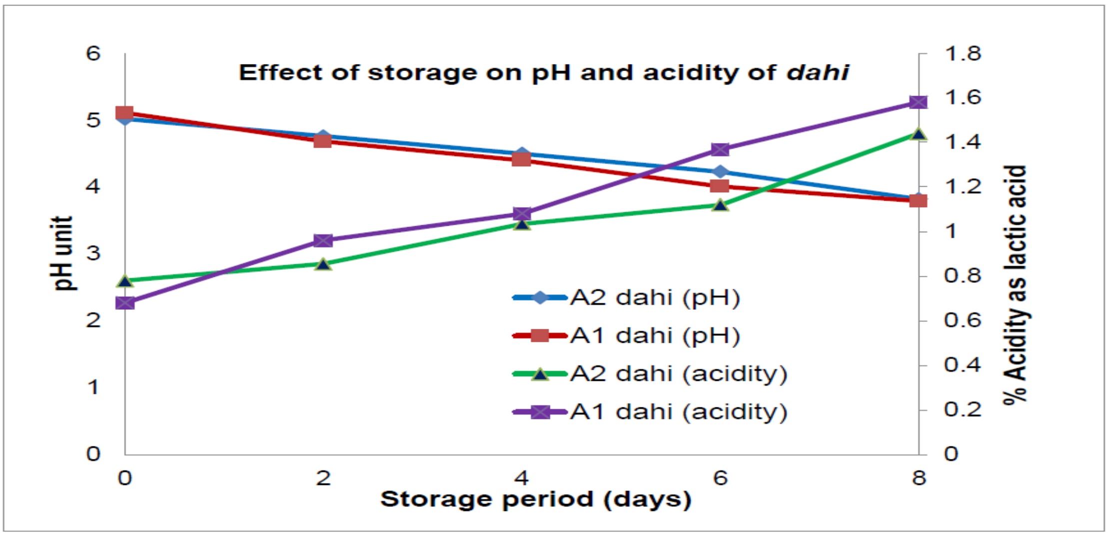


Fig 4.8. Acidity and pH of *dahi* prepared from A1 and A2 cow milk during storage at refrigerated condition

4.3.4. Measurement of the extent of proteolysis during storage of *dahi* prepared from the A1 and A2 cow milk

In order to evaluate the relationship between extent of proteolysis and release of BCM 7, proteolysis in *dahi* samples during storage was calculated through following parameters. The proteolysis was evaluated by measuring the changes in peptide content of water soluble extract and degree of hydrolysis of both types of *dahi* during storage period.

4.3.4.1. Peptide content in water soluble extracts (WSE) of *dahi* prepared from A1 and A2 cow milk

The protein contents in the filtrate obtain by 12% TCA is related to the peptide concentration in WSE of the *dahi*. The peptide concentrations (Fig 4.9) and in both type of *dahi* were increased with storage time. No significant difference was observed between peptide content of A1 and A2 *dahi* (P>0.05).

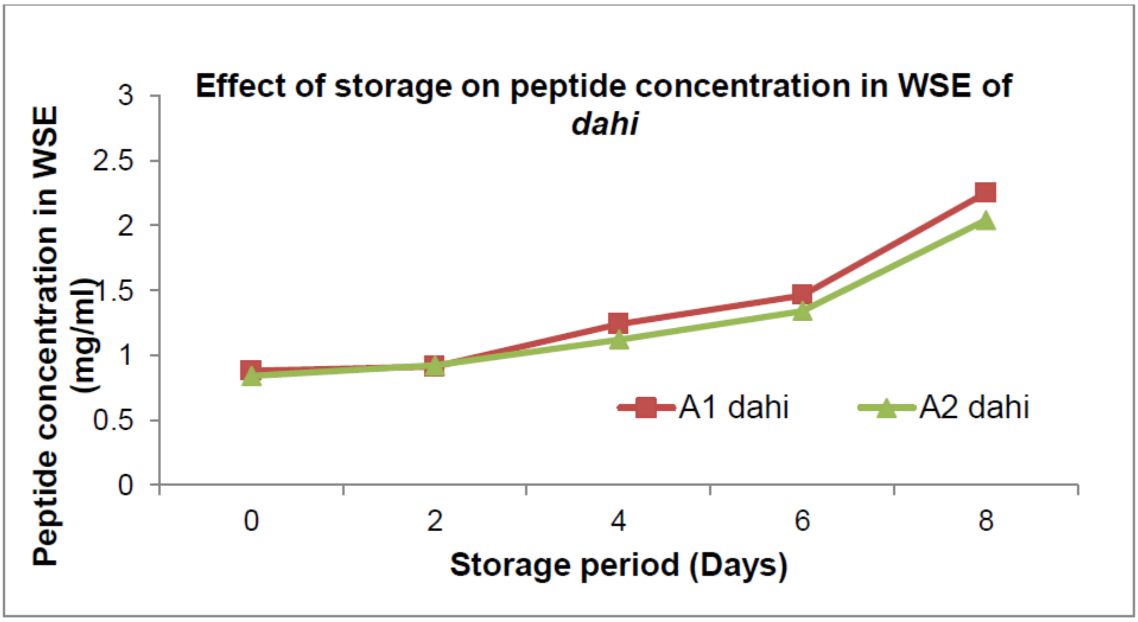


Fig. 4.9. Peptide content in water soluble extracts (WSE) of *dahi* prepared from A1 and A2 cow milk

4.3.4.2. Degree of Hydrolysis (DH) *of dahi* prepared from A1 and A2 cow milk

Extent of proteolysis in both types of *dahi* was determined by measuring therelease of free amino acid residues in terms of L-leucine content. The rate of proteolysis or release of peptides increased with storage period in both types of *dahi* (Fig. 4.10). Although there was no significant difference in the proteolytic activity between A2 and A2 *dahi* during storage at refrigeration condition (P>0.05). Schieber and Bruckner (2000) also reported extensive proteolysis of casein molecule during cold storage of yoghurt samples. Yoghurt prepared using Lactobacillus bulgaricus, L. acidophilus, L. casei, Bifidobacterium lactis showed significant increase in proteolysis during cold storage and hence increased in free amino acid content (Donkor *et al*., 2007a and 2007b). Similar findings reported by Otte *et al*., (2011) that stored yoghurt samples showed higher proteolysis compare to fresh yoghurt.

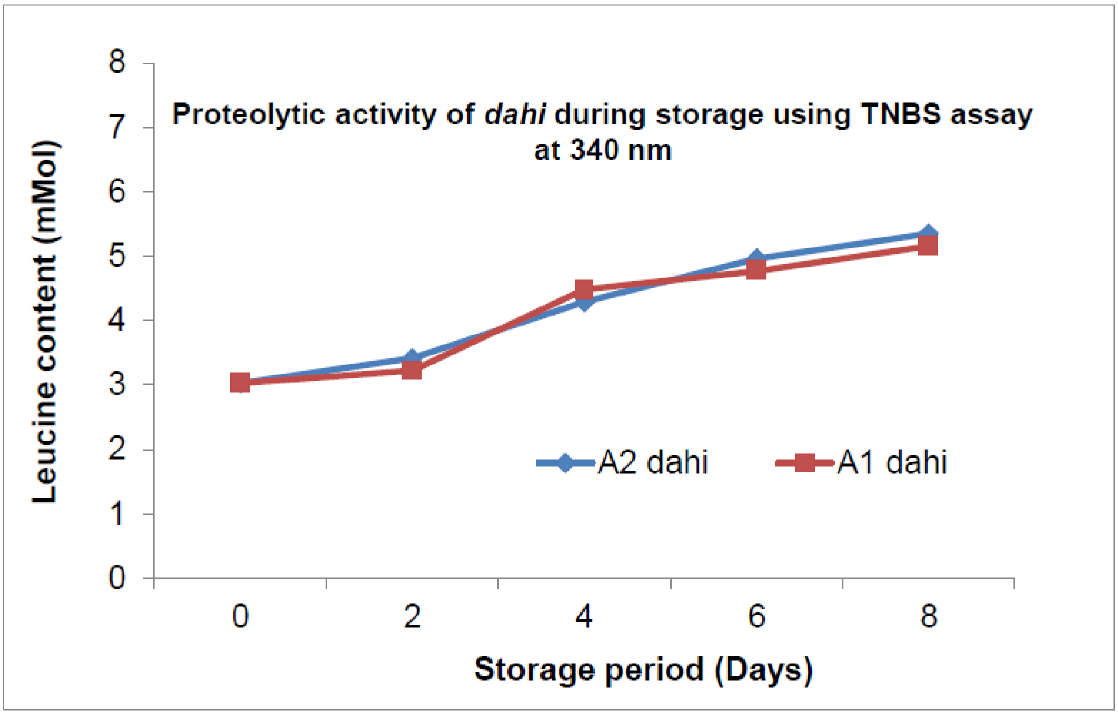


Fig 4.10. Degree of Hydrolysis (DH) *of dahi* prepared from A1 and A2 cow milk

4.3.5. PepX activity of *dahi* prepared from A1 and A2 cow milk

The PepX activities of prepared A1 and A2 *dahi* were showed in Fig. 4.11. There was an increase in PepX activity observed during storage. The increase in PepX activitywas 8.09-13.36 nmol/ ml/ min in A2 *dahi* and 7.86-12.90 nmol/ ml/ min in A1 *dahi* respectively. The enhanced activity may be due to growth of lactic acid bacteria as they are capable for multiplication even at low temperature (Donker *et al*., 2007a). Initially least increase of PepX activity observed to 2 days thereafter rapid increased in activity observed with decline pH in both types of *dahi*. This may be due to decrease in pH as reported by Atlan *et al.,* (1990) that with declination in pH value there was increased bacterial cell lysis resulting in release of bacterial entropeptidases into surrounding medium and there by enhanced PepX activity. Otte *et al*. (2011) also reported that low temperature of storage and low pH enhanced lysis of bacterial cells and released more PepX enzyme.

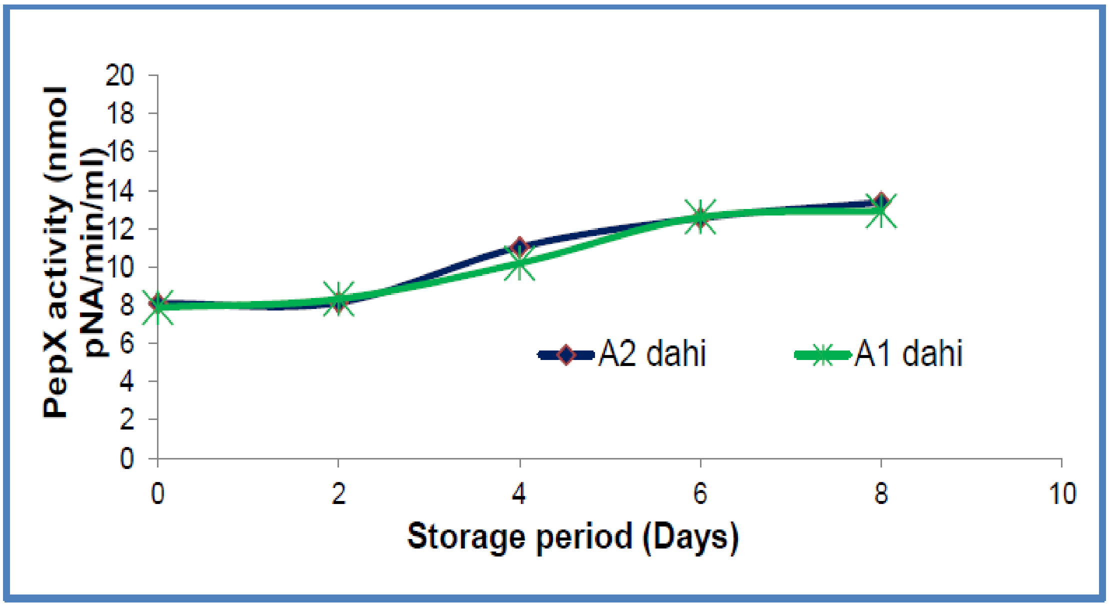


Fig. 4.11. PepX activity of *dahi* prepared from A1 and A2 cow milk

The increase in PepX activity was 8.09-13.36 nmol/ml/min in A2 *dahi* and 7.86-12.90 nmol/ml/min in A1 *dahi* respectively. The enhanced activity may be due to growth of lactic acid bacteria as they are capable for multiplication even at low temperature (Donker *et al*., 2007a). Initially least increase of PepX activity observed to 2 days thereafter rapid increased in activity observed with decline pH in both types of *dahi*. Thismay be due to decrease in pH as reported by Atlan *et al.,* (1990) that with declination in pH value there was increased bacterial cell lysis resulting in release of bacterial entropeptidases in to surrounding medium and there by enhanced PepX activity. Otte *et al*. (2011) also reported that low temperature of storage and low pH enhanced lysis of bacterial cells and released more PepX enzyme.

4.3.6. Quantification of BCM 7 in *dahi* and their gastrointestinal digest prepared from A1 and A2 milk by RP-HPLC method

The content of BCM 7 in *dahi* and in its gastrointestinal extracts during storage was shown in Table 4.10. BCM 7 was not identified in all samples of fresh and stored *dahi* up to one week (Fig 4.12 a and 4.13 a). Results pertaining to absence of BCM 7, inour dahi samples are in agreement with the findings of other similar studies in yoghurt by various researchers (De Noni and Cattaneo *et al*., 2010; Donkor *et al*., 2007a and 2007b; Gobbetti *et al*., 2000 and Schieber and Bruckner 2000). Storage of *dahi* at refrigerated condition did not have any impact on release of BCM 7. Schieber and Bruckner (2000) identified only pro BCMs in 3 week stored samples of yoghurt despite of extensive proteolysis of casein molecule during storage.

Even fermented milk produced by different strains of lactic acid bacteria able to generate numbers of pro BCMs but presence of BCM 7 was not found (Solieri *et al*., 2018; Plaisancie *et al*., 2013; Kunda *et al*., 2012; Otte *et al*., 2011). Release of casomorphins in fermented dairy products is considered unlikely because lactic acid bacteria used in preparation of fermented products exhibit X-propyl-dipeptidyl-amino peptidase activity. Thereby if any casomorphins generated may be further degradated to simpler peptides and amino acids (Donkor *et al*., 2007b). Paul and Somkuti (2009) also reported that, fermentation with *L. delbrueckii* ssp. *bulgaricus* causes significant hydrolyses of antimicrobial and hypertensive peptides after 4 hours incubation at acidic condition. This study suggested that if any BCMs generated during fermentation may be degraded during cold storage of the fermented dairy products. These results indicate that lactic acid bacteria used for preparation of *dahi* do not have the ability to generate BCM 7. Presence of BCM 7 in fermented products are contradictory as some studies reported BCM 7 in yoghurt, probiotics drinks (Jarmołowska and Krawczuk 2012; Nguyen *et al*., 2014). BCM 7 was also isolated from kefir a yeast fermented milk product by Dallas *et al*. (2016), although the type of milk used for preparation of kefir was not specified by the author.

Table 4.10. Quantification of BCM 7 in *dahi* and their gastrointestinal digestprepared from A1 and A2 milk by RP-HPLC method

	Fresh *Dahi* (0 Days)		**After 2 Days**		**After 4 Days**		**After 6 Days**	
	A1	**A2**	**A1**	**A2**	**A1**	**A2**	**A1**	**A2**
Control	nd	nd	nd	nd	nd	nd	nd	nd
Salivary Extract	nd	nd	nd	nd	nd	nd	nd	nd
Gastric Extract	<LOQ	<LOQ	<LOQ	<LOQ	nd	<LOQ	nd	<LOQ
Intestinal extracts(ng/g)	896.51± 152.08	191.40± 30.56	857.11± 136.62	160.26± 27.80	440.07± 70.37	82.35± 13.55	281.35± 172.57	<LOQ

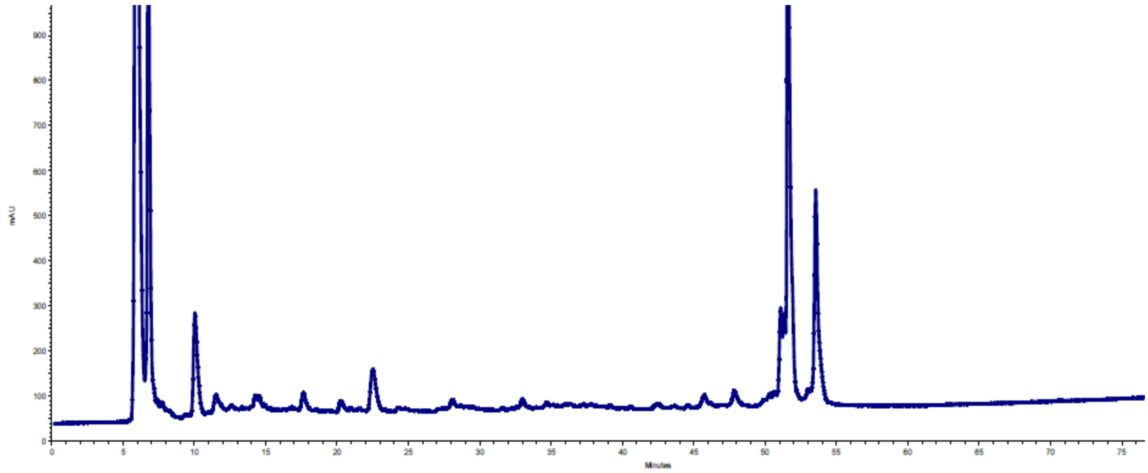
(Values are mean ± SD n=8, <LOQ- below limit of quantification)

Dahi prepared from A1 and A2 cow milk subjected to simulated *in-vitro* digestion to study the effect of fermentation and gastrointestinal conditions on fate of BCM 7.Salivary extracts of all samples did not contain BCM 7 as depicted in Table 4.10. It is well expected that no proteolysis in oral phase so release of BCM 7 was not observed (4.12 b and 4.13 b). During gastric digestion release of BCM 7 was observed in both type of *dahi* prepared with A1 and A2 cow milk (Fig 4.12 c and 4.13 c). In our previous section of study (Table 4.6 and 4.7) MS analysis confirmed that along with BCM 7, a number of BCM 7 like peptides such as BCM-8, BCM-9 were released during simulated gastrointestinal digestion of A1 and A2 raw milk. It is better to refer as BCM 7 like peptides instead of BCM 7 in reporting the RP-HPLC results. Concentration of BCM 7 like peptides was very low and unable to quantify by RP-HPLC method (Table 4.10) in gastric extracts of both types of dahi (A1 and A2). During storage it was observed that content of BCM 7 like peptides were decreased in both type of dahi and disappeared in A2 *dahi* after 4 days of storage whereas in A1 *dahi* presence of these peptides observed until 6 days of storage. Very few studies were carried out to see the effect of fermentation process in release of BCM 7. Cieślińska *et al*., (2007) also reported the release of BCM 7 during peptic digestion of A1 and A2 raw milk. Release of BCM 7 like peptides during gastric digestion probably be the resultant of complex biochemical reaction occurred due to acidic pH, action of pepsin along with bacterial activity. These peptides may be further breakdown to smaller peptides by lactic acid bacteria resulting in release of BCM 7. It has been reported previously that proteolytic systems of lactic acid bacteria can hydrolyze 35-40 % peptides bonds in β-CN molecule (Solieri *et al*., 2018, Gobbetti *et al*., 2000). Authors also revealed that cleavage sites were distributed throughout the entire sequence of β-CN molecule and preferred sites of cleavage are Ser, Ala, Asn and His, Met, Arg, Aps, Tyr resulting in the release of BCM 7. Chabance *et al*., (1998) also mentioned that gastric digestion of yoghurt released more peptides compared to milk samples. Also fermentation process able to changed the structure of milk proteins present in *dahi* causing a change in digestion pattern of the products. During storage disappearance of BCM 7 peptides in *dahi* might be the further degradation of the peptide by amino peptidases of the culture (Donkor *et al*., 2007b).

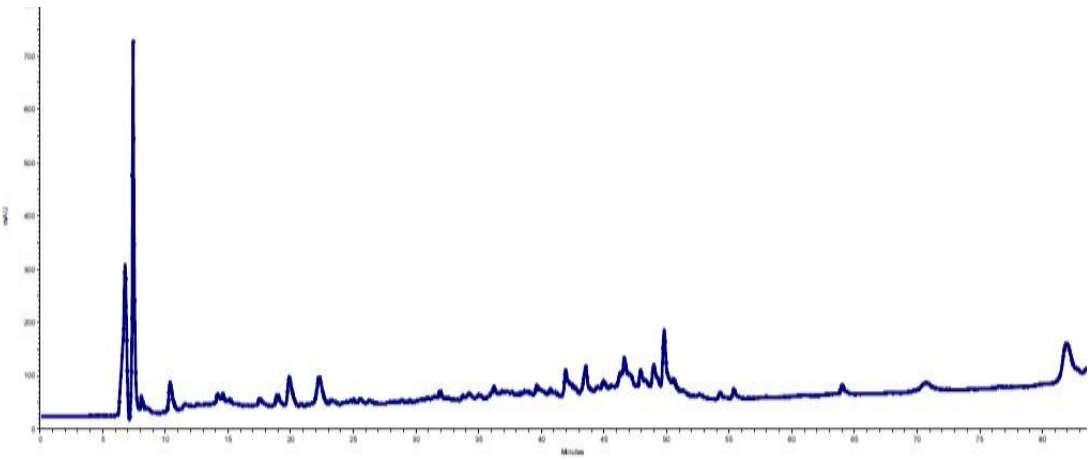
As shown in Table 4.10 and 4.11 during simulated *in-vitro* gastrointestinal digestion of A2 and A1 *dahi*, BCM 7 like peptides were detected in all samples Fig 4.12 d and 4.13 d). The concentrations were significantly higher in A1 *dahi* than A2 *dahi* ($P<0.05$). About 4-5 times higher content of BCM 7 like peptides was observed in A1 *dahi* (281.35- 896.51ng/ ml) than A2 *dahi* (82.35-191.40 ng/ ml). Similar trend was observed in our previous study conducted on processed milk samples. Findings of our previous study (Table 4.5) showed that BCM 7 like peptides were 97.44 ng/ml , 22.54 ng/ml in raw milkand boiled milk respectively however in A1 raw milk it was 1399.32 ng/ml and 372.54 ng/ml in A1 boiled milk. On comparing BCM 7 like peptides in *dahi* with milk, it was observed that BCM 7 concentration in *dahi* nearly similar to the raw milk but higher thanboiled milk. It may be due to raw milk consist of natural micro flora so proteolysis were similar to *dahi*. However in boiled milk heating may cause degradation of BCM 7 but in *dahi* lactic acid bacteria may be responsible for the proteolysis and generation of higher concentration of BCM 7 like peptides.

MS analysis in our previous section (Table 4.6, 4.7 and 4.8) reported that numberof BCM 7 like peptides such as BCM 7 + 1 (**^{59}VYPFPGPI^{66})** and BCM + 2 (**^{58}LVYPFPGPI^{66}**) were identified from A2 milk after gastrointestinal digestion. Other A2 milk derived pro BCM peptides such as β (59-66), β (58-66), β (57-68), β (56-68), β (55-68) were identified in intestinal extracts. In spite of release of BCM 7 like peptides, BCM 7 (β CN- 60-66) was not identified after *in-vitro* gastrointestinal digestion of A2 milk. Where as in A1 milk, BCM 7 (**^{60}YPFPGPI^{66}**) and BCM 7 like peptides such as **^{59}VYPFPGPI^{66}**, **^{53}AQTQSLVYPFPGPI^{66}** and **^{54}QTQSLVYPFPGPI^{66}** were released after simulated gastrointestinal digestion.

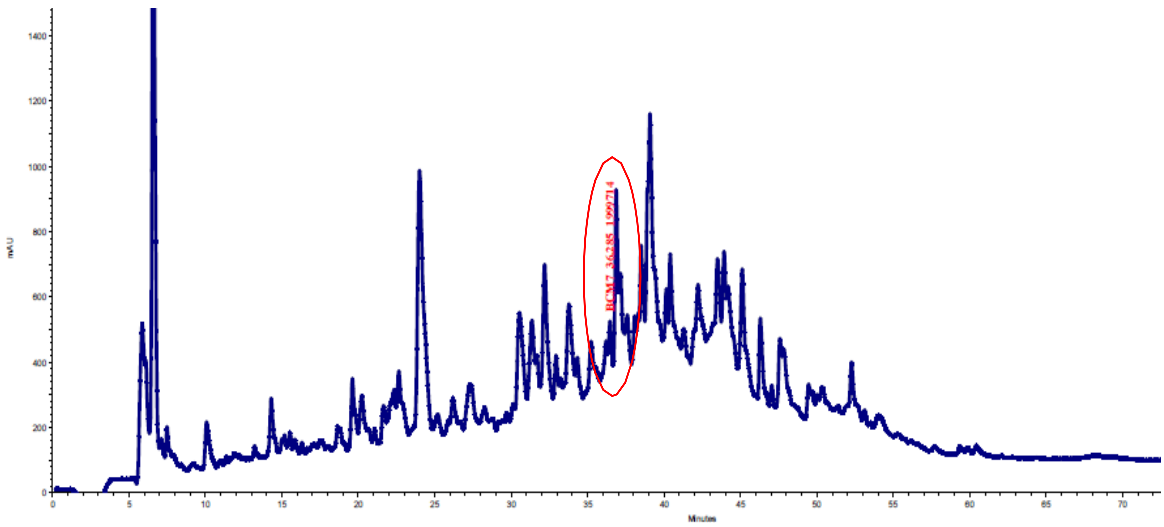
De Noni and Cattanea (2010) also reported the release of BCM 7 during gastrointestinal digestion of fermented probiotic milk and yoghurt. These dairy products were commercially prepared with the mixed milk containing A1, A2 and B variants. Very few studies are available related to BCM 7 release during digestion of fermented milk. Data available for milk showed that intestinal extract of A1 milk reported to contain higher amount of BCM 7 than A2 milk (Cieslinka *et al*., 2012, Fiedorowicz *et al*., 2014, Asledottir *et al*., 2017 and 2018). Ul Haq *et al*., (2015) also reported that on enzymatic hydrolysis of purified β-CN fraction of Karan Fries cow milk higher concentration of BCM 7 released from A1 than A2 variant. Higher concentration of BCM 7 in A1 *dahi* samples is related to β-CN sequence specific to amino acid residue 'His' at position "67". The peptide bond between Ile_{66}-His_{67} in A1 variant is readily hydrolysed by gastrointestinal enzymes than the bond between Ile_{66}-Pro_{67} of the A2 variant. Under normal digestive condition A2 beta-casein releases negligible amount of BCM 7 (Jinsmaa and Yoshikawa 1999; Cieślińska at al., 2007; De Noni 2008). Contradictory to these findings, recently Nguyen *et al*. (2020) reported that that gastrointestinal digestion did not released any BCM 7 or pro BCMs in commercial yoghurt. Authors also identified the cleavage sites of β-CN during digestion and mentioned that most of peptides released were from the 91- 111 region of the molecule.



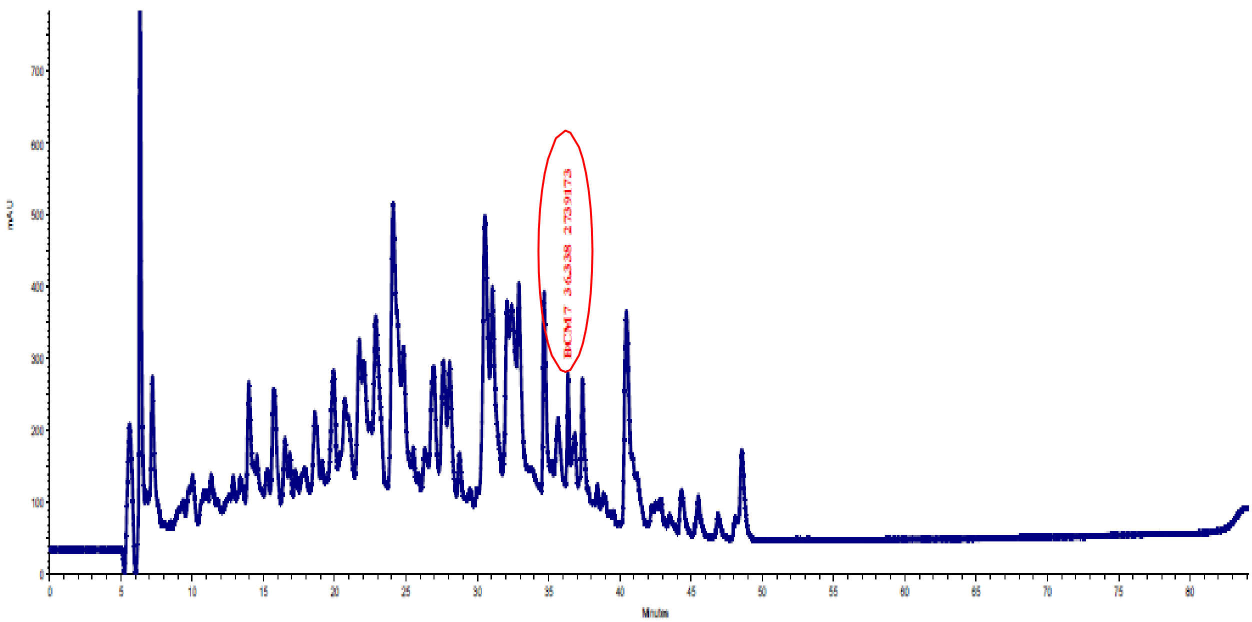
(a) RP-HPLC chromatogram of A1 *dahi* (fresh)



(b) RP-HPLC chromatogram of A1 *dahi* after salivary digestion

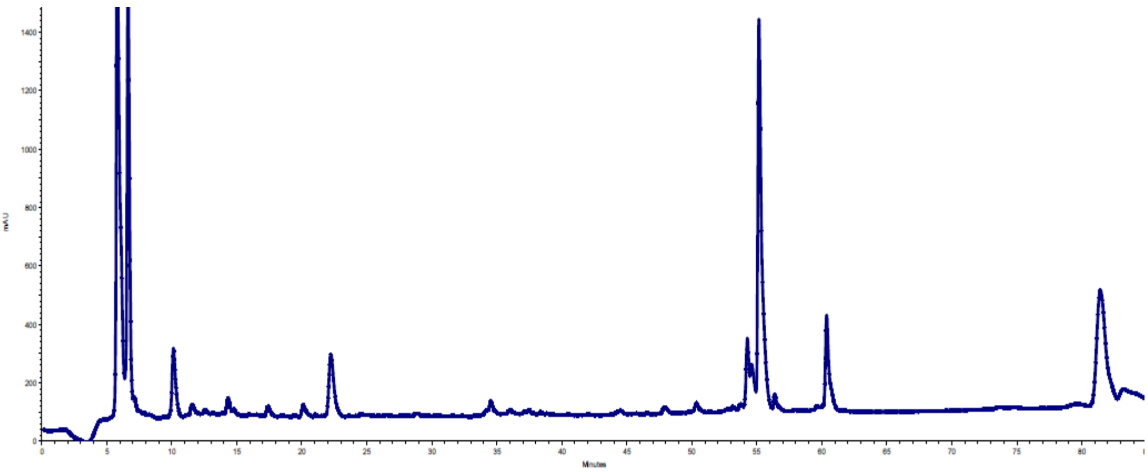


(c) RP-HPLC chromatogram of A1 *dahi* after gastric digestion (release of BCM 7 in red marking)

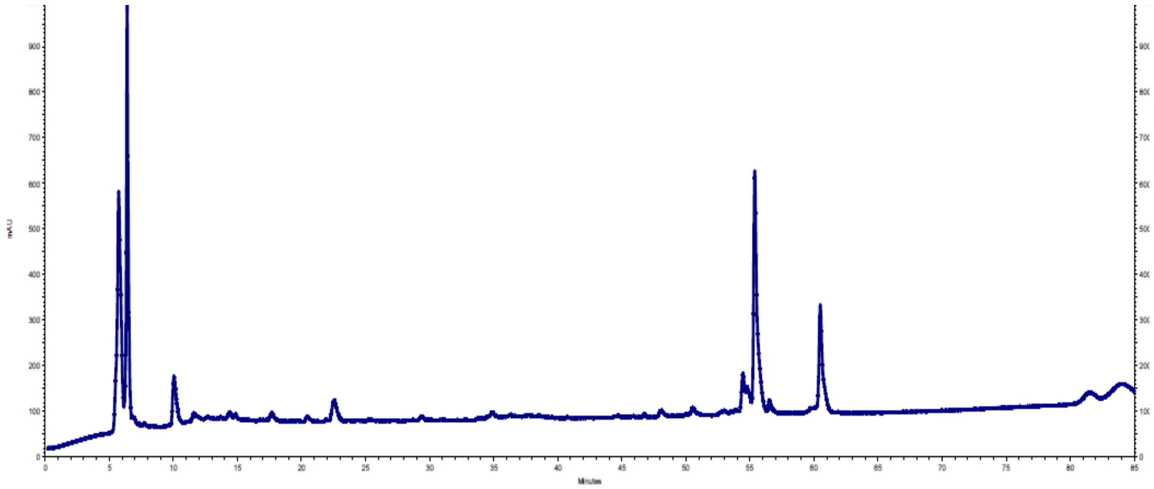


(d) RP-HPLC chromatogram of A1 *dahi* after intestinal digestion (release of BCM 7 in red marking)

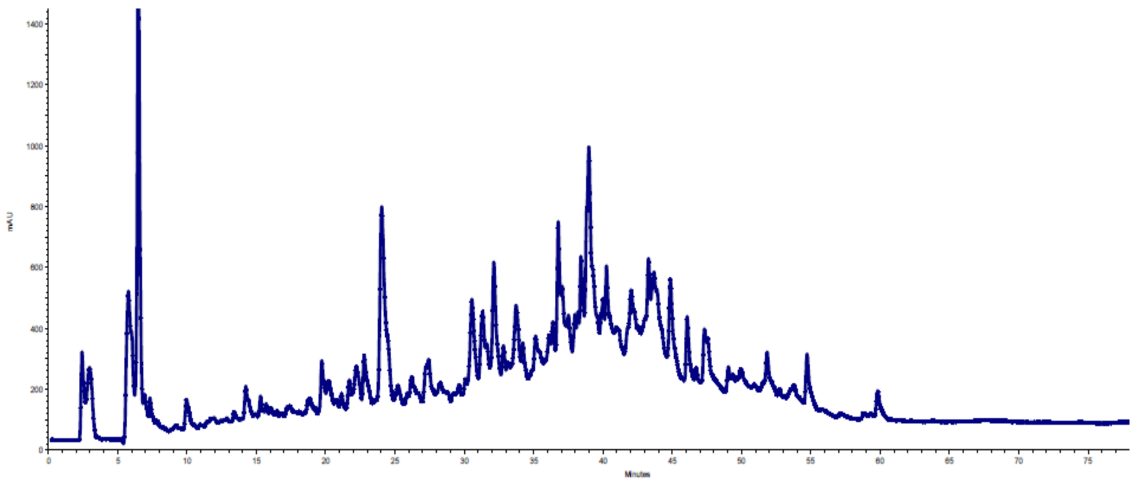
Fig. 4.12 (a-d) Typical RP-HPLC chromatogram of A1 *dahi* after different stages of simulated *in-vitro* digestion



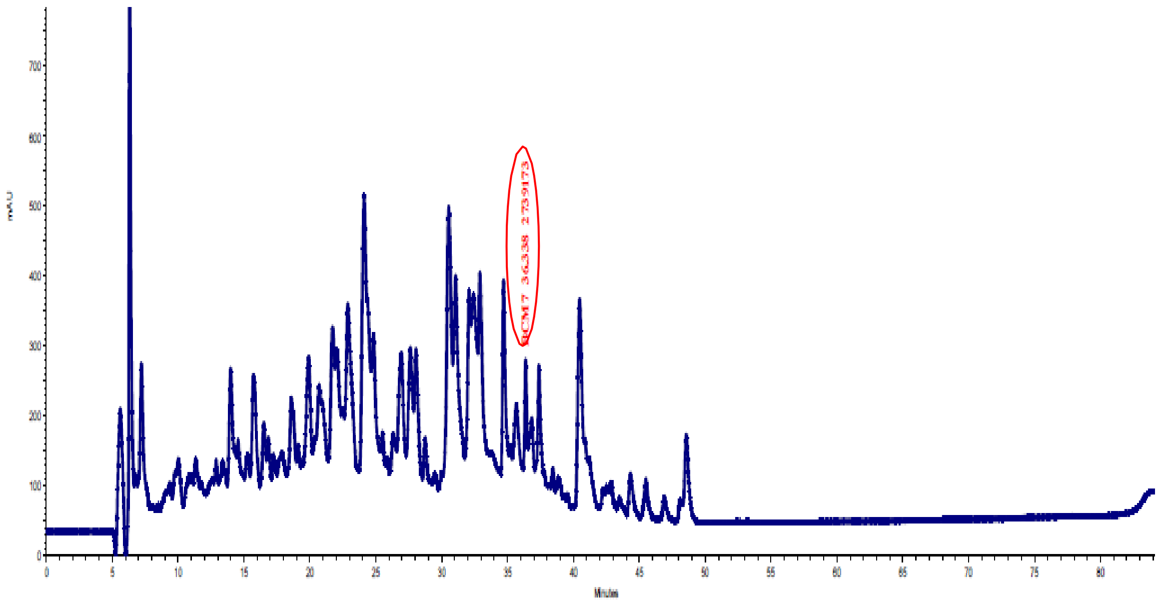
(a) RP-HPLC chromatogram of A2 *dahi* fresh



(b) RP-HPLC chromatogram of A2 *dahi* after salivary digestion



(c) RP-HPLC chromatogram of A2 *dahi* after gastric digestion



(d) RP-HPLC chromatogram of A2 *dahi* after intestinal digestion (release of BCM 7 in red marking)

Fig. 4.13 (a-d) Typical RP-HPLC chromatogram of A2 *dahi* after different stages of simulated *in-vitro* digestion

4.3.7. Quantification of BCM 7 in *Dahi* and their gastrointestinal digest prepared from A1 and A2 milk by ELISA assay

ELISA technique was used to cross validate the results obtained by RP-HPLC method for quantification of BCM 7 in *dahi* samples (Table 4.11). BCM 7 like peptides were identified in gastric extracts of both types of *dahi* samples by ELISA assay. The concentration of BCM 7 in gastric extracts varied 10.00-18.0 ng/ml and 12.00-16.00 ng/ml in *dahi* prepared from A2 and A1 milk respectively*.* There was no significant difference in the release of BCM 7 with respect to the types of variants. Similarly Cieślińska *et al*. (2012) and identified the release of BCM 7 in peptic extracts of A2 and A1 raw milk. Despite of presence of BCM 7 in all gastric samples we were unable to quantify with RP-HPLC method being low sensitivity of the method compare to ELISA. Surprisingly RP-HPLC was able to provide the evidence of presence of BCM 7 like peptides in *dahi* samples (Table 4.11).

Table 4.11. Comparison of ELISA and RP-HPLC method for quantification of BCM7 in digestive extracts of *dahi* prepared with A1 and A2 milk

Storage period (Days)	A1 *dahi* (ng / ml)		A2 *dahi* (ng / ml)	
	ELISA	RP-HPLC	ELISA	RP-HPLC
Gastric Extract				
Fresh	10.0 ± 6.24	<LOQ	14.0 ± 4.60	<LOQ
2 Days	13.08 ± 4.78	<LOQ	12.0 ± 8.40	<LOQ
4 Days	18.04 ± 4.12	<LOQ	16.0 ± 6.48	nd
6 Days	11.0 ± 3.66	<LOQ	14.0 ± 5.00	nd
Intestinal Extract				
Fresh	590.26 ± 18.64	896 ± 152.82	166.87± 10.24	191.75 ± 30.54
2 Days	528.00 ± 28.18	856.75 ± 136.64	132.93 ± 11.66	160.00 ± 27.98
4 Days	222.26±28.16	440.00 ± 70.39	55.55 ± 2.42	82.50 ± 13.64
6 Days	133.64±4.32	218.25 ± 172.48	24.97 ± 8.60	nd

Values are mean ± SD n=8, <LOQ- below limit of quantification

Simulated *in-vitro* intestinal extracts of all samples were analyzed for BCM 7 by ELISAassay (Table 4.11). Similar trend of release of BCM 7 after intestinal digestion was observed in both by RP-HPLC and ELISA. Concentrations of BCM 7 obtained by ELISA assay were significantly lower than the concentration evaluated by RP-HPLC method. Concentration of BCM 7 in fresh A2 *dahi* intestinal digestive extracts was 166.87 ng/ml and 191.75 ng/ml as calculated by ELISA and RP-HPLC method respectively. In case of intestinal digestive extracts of A1 *dahi* BCM 7 concentration evaluated by ELISA and RP-HPLC was 590.26 and 896.00 ng/ml respectively.

Difference in the concentration of BCM 7 in both methods was due to presence of number of BCM 7 like peptides in digestive extracts having similar physicochemical and absorption characteristics. There by providing more absorption in RP-HPLC method and indicated higher concentration. Whereas

ELISA assay is specific to BCM 7 peptide and least interference by other similar peptides.

When stored samples were analyzed for BCM 7 after intestinal digestion by RP-HPLC and ELISA methods, it was observed that concentration of BCM 7 like peptides decreased with storage time significantly (P>0.05) (Fig 4.14). There was no significant difference up to 2 days of storage. On 4th day of storage there was significant decrease of BCM 7 observed in all samples, whereas on 6th day of storage BCM 7 like peptides were identified only in the samples of A1 *dahi*. On 8th day pH of stored sample were very low near to 3.80 ± 0.12 in all samples with acidity 1.45 ± 0.10% and samples were showing syneresis and mould growth so further analysis for quantification of BCM 7 not carried out. Findings of the present study revealed that BCM 7 decreased with storage time. Similar results were reported by Jarmołowska and Krawczuk (2012) in yoghurt and probiotics that concentration of BCM 7 decreased on storage. The reason of decrease in content of BMC 7 during storage of fermented dairy products is the PepX activity of lactic acid bacteria. BCM 7 is a proline rich peptide more resistant to digestive enzymes but highly susceptible to LAB derived PepX that preferentially cleaves peptide bonds involving proline. Atlan *et al*., (1990) reported that PepX is present in the cytoplasm and releases into the surrounding medium on destruction of bacteria cells. In our study we found that PepX activity increased with storage time (Fig. 4.11). At low pH and low temperature storage condition of yoghurt, bacterial cells are more prone to lysis and release more PepX (Otte *et al*., 2011). Hence decrease in BCM 7 during storage of *dahi* is reported. Nielsen *et al*., (2009) also reported that proteases present on cell wall of lactic acid bacteria hydrolysed caseins into larger peptides, and further hydrolysis takes place into di and tri-peptides inside the bacterial cell by endo peptidases. Consequently, LAB may able to cleave β-CNs into BCM 7 and other related BCMs during fermentation and subsequently degrade them into even smaller peptides during storage. Kunda *et al*., (2012) identified a number of fragmented peptides of BCM 7 such as β-CN (60-61) and β-CN (62-63) along with pro BCMs in yoghurt. These findings indicate that pro BCMs and BCM 7 can further hydrolysed to smaller peptides. Paul and Somkuti (2009) also reported that, fermentation with *L. delbrueckii* ssp. *Bulgaricus,* causes significant hydrolyses of antimicrobial and hypertensive peptides after 4 h incubation at pH 4.5. This study suggests that any BCMs generated during fermentation may be degraded during storage at low temperature and pH (4.5). Several studies reported that proteases and peptidases of LAB remained active throughout fermentation and post-storage in fermented dairy products (Farahat *et al*., 2013). Recently Nguyen *et al*. (2018) studied the effect of fermentation on BCM 7 in UHT milk. Sample was spiked with BCM 7 peptide and fermentation was carried out using *Lactobacillus delbrueckii* subsp.*bulgaricus* and *S. thermophilus* bacteria. The findings revealed that lactic acid bacteria used in study were able to degrade entire added peptide at low pH condition. Hence this evidence suggested that lactic acid bacteria have ability to degrade BCM 7 peptide in fermented products.

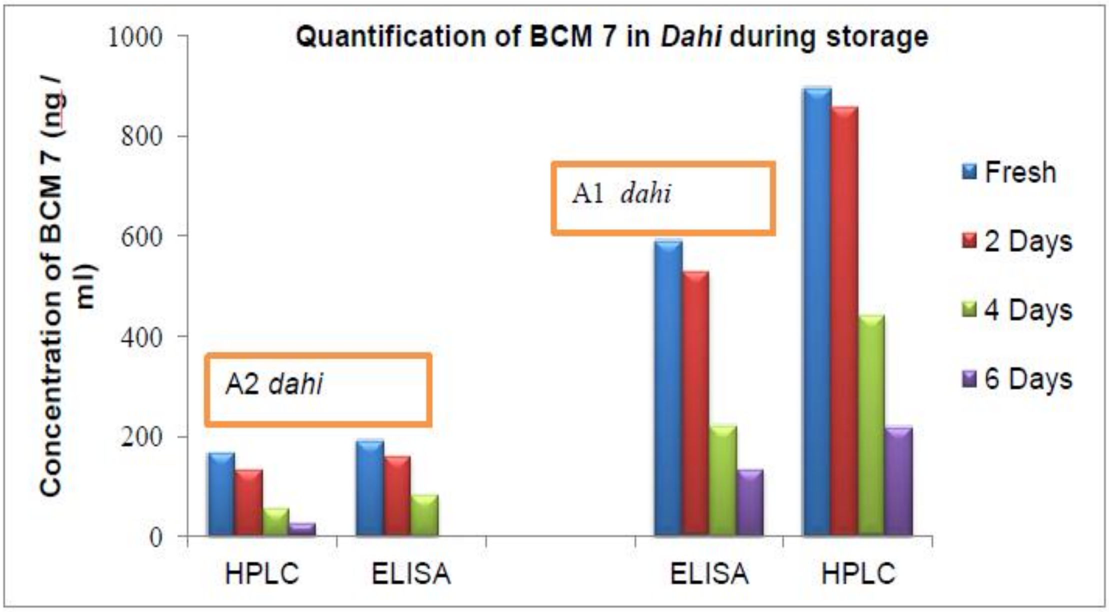


Fig 4.14. BCM 7 content in intestinal extracts of A1 and A2 *dahi* during storage at refrigerated temperature

There was no significant difference up to 2 days of storage. On 4th day of storage there was significant decrease of BCM 7 observed in all samples, whereas on 6th day of storage BCM 7 like peptides were identified only in 2 samples of A1 *dahi.* On 8th day pH of stored sample were very low near to 3.80 ± 0.12 in all samples with acidity 1.45 ± 0.10% and samples were showing syneresis and mould growth so further analysis for quantification of BCM 7 not carried out. Findings of the present study revealed that BCM 7 decreased with storage time. Similar results were reported by Jarmołowska and Krawczuk (2012) in yoghurt and probiotics that concentration of BCM 7 decreased on storage. The reason of decrease in content of BMC 7 during storage of fermented dairy products is the PepX activity of lactic acid bacteria. BCM 7 is a proline rich peptide more resistant to digestive enzymes but highly susceptible to LAB derived PepX that preferentially cleaves peptide bonds involving proline. Atlan *et al*., (1990) reported that PepX is present in the cytoplasm and releases into the surrounding medium on destruction of bacteria cells. In our study we found that PepX activity increased with storage time (Fig. 4.11). At low pH and low temperature storage condition of yoghurt, bacterial cells are more prone to lysis and release more PepX (Otte *et al*., 2011). Hencedecrease in BCM 7 during storage of *dahi* is reported. Nielsen *et al*., (2009) also reported that proteases present on cell wall of lactic acid bacteria hydrolysed caseins into larger peptides, and further hydrolysis takes place in to di and tri-peptides inside the bacterial cell by endo peptidases. Consequently, LAB may able to cleave β-CNs into BCM 7 and other related BCMs during fermentation and subsequently degrade them into even smaller peptides during storage. Kunda *et al*., (2012) identified a number of fragmented peptides of BCM 7 such as f (60-61) and f (62-63) along with pro BCMs in yoghurt samples. These findings indicate that pro BCMs and BCM 7 can further hydrolysed to smaller peptides. Paul and Somkuti (2009) also reported that, fermentation with *L. delbrueckii* ssp. *Bulgaricus* causes significant hydrolyses of antimicrobial and hypertensive peptides after 4 h incubation at pH 4.5 condition. This study suggests that any BCMs generated during fermentation may be degraded during cold storage at pH 4.5. Several studies reported that proteases and peptidases of LAB remained active throughout fermentation and post-storage in fermented dairy products (Farahat *et al*., 2013). Recently Nguyen *et al*. (2018) studied the effect of fermentation on BCM 7 in UHT milk. Sample was spiked with BCM 7 peptide and fermentation was carried out using *Lactobacillus delbrueckii* subsp. *Bulgaricus* and/or *S. thermophilus* bacteria. The findings revealed that lactic acid bacteria used in studywere able to degrade entire added peptide at low pH condition. Hence this evidence suggested that lactic acid bacteria have ability to degrade BCM 7 peptide in fermented products.

4.4. Effect of ripening on the release of BCM 7 in Cheddar cheese and in their simulated *in-vitro* digestive extracts prepared from milk of A1 and A2 cow milk

4.4.1. Preparation of Cheddar cheese from A1 and A2 milk

Four batches of Cheddar cheese were prepared as per standard procedure provided in Fig 3.6, from each A1 and A2 milk. Bovine chymosin (rennet) from Hansen (Denmark) was used as coagulating agent.

4.4.2. Physicochemical analysis of Cheddar cheese prepared from A1 and A2 milk

To evaluate the difference in basic composition of cheese prepared with A1 and A1 milk, proximate analysis was carried out (Table 4.12). Analysis revealed that moisture decreased with increase in ripening. Initially first 4 months of ripening, decrease in moisture was not significant in both types of Cheddar cheeses prepared from A1 and A2 milk. After, after 5 months changes in moisture content was significant in both types of cheese. Reduced moisture content in Cheddar cheese leads to increase in the levels of total protein and salt content. However fat content decreases with ripening time in both types of cheese. Reduction in fat content may be due to biochemical changes such as lipolysis by microbial culture and formation of volatile flavoring compounds (McSweeney2004). Similar trend were also observed by Khan *et al*., (2019) and Mezo-Solis *et al*. (2020) that moisture and fat decreases during ripening. As far as A1 and A2 milk concern used for the preparation of Cheddar cheese, all batches of cheese showed similar results and no significant difference was observed between proximate compositions of both types of cheeses (($p>0.05\%$).

The experimental Cheddar cheese samples were analyzed for mineral such as sodium, calcium, potassium and phosphorous content using atomic absorption spectrophotometer (Table 4.13). Content of all mineral remained nearly constant throughout ripening and no significant changes occurred during ripening period ($p>0.05\%$). Mezo-Solis *et al*., (2020) reported that there was no change in ash content in cheese during ripening. Among all minerals, highest content of calcium was observed in both Cheddar cheeses followed by sodium, phosphorous and sodium.

Table 4.12. Physicochemical analysis of Cheddar cheese prepared from A1 andA2 milk

Storage Period (Months)	Moisture (%)		Protein (%)		Fat (%)		Salt Content (%)	
	A1	**A2**	**A1**	**A2**	**A1**	**A2**	**A1**	**A2**
Fresh	37.29±0.20	37.24±0.08	26.26±0.16	25.90±0.12	33.12±0.24	33.87±0.08	1.95±0.06	2.02±0.02
1	36.98±0.12	36.52±0.40	26.24±0.20	25.94±0.08	33.14±0.16	33.68±0.10	2.12±0.08	1.98±0.06
2	36.56±0.14	35.94±0.010	26.22±0.08	25.84±0.11	32.88±0.34	32.96±0.12	2.18±0.04	1.94±0.12
3	35.87±0.17	35.66±0.10	26.92±0.12	25.88±0.14	32.66±0.22	32.84±0.10	2.24±0.12	2.14±0.12
4	35.28±0.11	34.56±0.18	27.16±0.16	25.96±0.12	32.26±0.12	32.06±0.18	2.08±0.10	2.18±0.12
5	34.08±0.17	33.86±0.30	27.56±0.12	26.04±0.22	32.68±0.16	32.54±0.24	2.22±0.08	2.22±0.04
6	33.17±0.12	33.02±0.26	27.84±0.04	26.80±0.26	32.59±0.26	32.02±0.16	2.38±0.10	2.29±0.08
7	32.92±0.08	32.82±0.22	28.08±0.14	26.95±0.14	32.06±0.14	31.86±0.22	2.46±0.08	2.26±0.18
8	32.07±0.24	31.99±0.06	28.29±0.26	27.12±0.12	31.80±0.26	31.66±0.24	2.54±0.14	2.26±0.12
9.	31.57±0.24	30.99±0.06	28.16±0.26	26.12±0.12	30.80±0.26	29.66±0.24	2.26±0.14	2.24±012

(values are mean ± SD, n=4)

Table 4.13. Mineral content in A1 and A2 Cheddar cheese during ripening

Storage Period (Months)	Sodium (% mg)		Calcium (% mg)		Potassium (% mg)		Phosphorous (% mg)	
	A1	**A2**	**A1**	**A2**	**A1**	**A2**	**A1**	**A2**
Fresh	813.26±4.62	816.94±6.26	922.26±8.16	911.90±10.12	135.12±8.24	135.87±6.08	748.24±10.06	756.02±8.02
1	836.98±10.12	812.52±3.62	926.94±0.20	927.64±8.08	132.14±10.16	138.68±4.10	744.96±6.08	760.12±10. 26
2	811.18±8.14	825.94±4.01	926.22±4.08	907.04±6.34	134.06±6.34	135.16±10.12	738±6.24	752.94±6.296
3	805.82±12.16	799.66±12.26	925.92±10.12	926.88±8.14	136.66±10.22	134.84±6.10	756.80±3.12	760.26±4.68
4	816.28±6.122	814.56±3.68	904.16±8.16	925.96±6.12	136.26±8.12	132.06±6.22	762.80±0.10	758.26±8.60
5	814.08±8.62	823.86±10.30	923.96±0.12	924.04±10.36	133.68±6.16	136.54±7.56	772.0±12.08	758.62±3.68
6	823.17±9.16	826.02±0.36	913.34±6.22	924.80±6.26	142.59±8.26	143.02±8.16	759.68±10.12	765.08±10.08
7	832.92±6.26	832.82±8.22	928.08±8.14	923.95±6.14	140.06±4.14	142.36±6.28	761.60±8.12	764.00±9.18
8	812.07±8.24	831.99±10.06	932.90±6.86	923.12±0.12	141.80±10.26	142.88±7.24	764.54±8.26	766.26±8.36
9	831.12±6.26	830.95±8.18	933.18±8.24	922.98±10.04	142.28±10.22	143.18±10.14	768.42±8.26	768.32±8.18

Values are mean ± SD, n=4)

4.4.3. Effect of ripening on acidity and pH of the Cheddar cheese prepared fromA1 and A2 milk

Ripening had significant effect on acidity and pH of Cheddar cheese. Acidity increased with storage period up to 5 months of storage (Fig 4.15). After six months there was slight decrease in acidity observed.

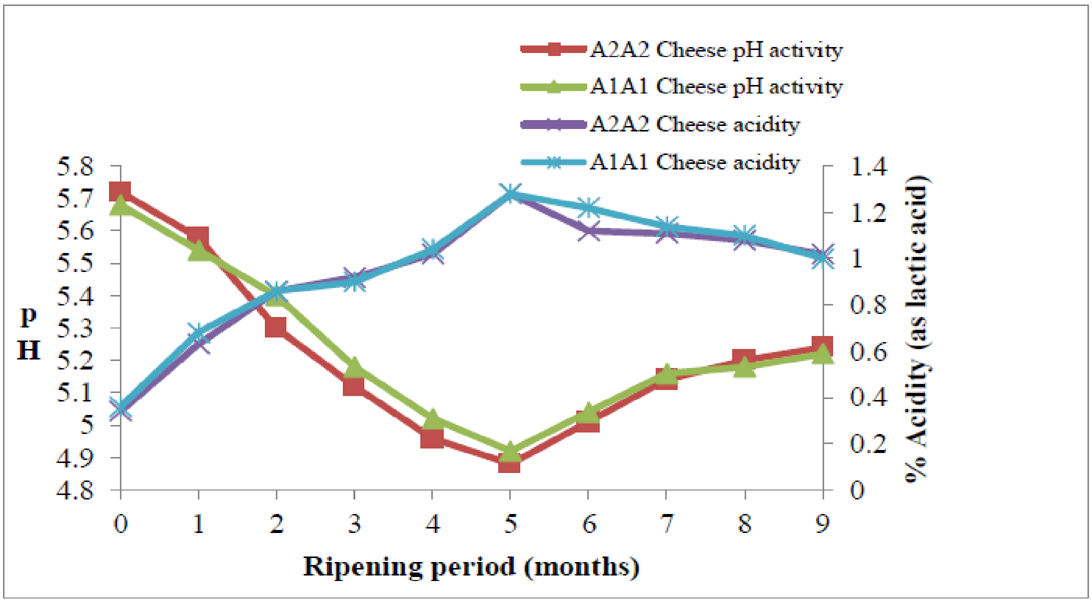


Fig. 4.15. Effect of ripening on acidity and pH of the Cheddar cheese prepared with A1 and A2 milk

Ripening had significant effect on acidity and pH of Cheddar cheese ($p<0.05\%$). Acidity increased upto 5 months of storage period (Fig 4.15). After six months there was slight decrease in acidity observed in both types of Cheddar cheeses. Initial increase in acidity was due to production of lactic acid from lactose and activity of starter culture, milk proteases as residual lactose is metabolized rapidly to lactate during the early stages of ripening (Azarnia *et al*., 2006, Ramzan *et al*., 2010, Mamo 2017). The rate and extent of acid production in Cheddar cheese determined the quality of the finish product. There was gradual decreased in pH observed in both types of Cheddar cheese during ripening up to 5 months (Fig 4.15) due to production of lactic acid by selected cultures of lactic acid bacteria (LAB) used in cheese making. After 5 months of ripening there was increased in pH value, due to decreased in acidity as residual lactose got exhausted by LAB and complex biochemical changes during ripening (Sousa, Ardo and McSweeney 2001).

4.4.4. Degree of proteolysis during ripening of A1 and A2 Cheddar cheese Degree of proteolysis of A1 and A2 Cheddar cheese during ripening

To study the relationship between rate of proteolysis and formation of BCMs, extent of proteolysis was estimated by determining the ripening index to know the extent of primary and secondary proteolysis in Cheddar cheese prepared from A1 and A2 milk. The primary proteolysis was calculated by determining the ratio of nitrogen content of water soluble nitrogen to total nitrogen of cheese. Primary proteolysis indicates the release of large molecular weight peptides from caseins by the action of rennet (chymosin), milk enzymes and proteinases from starter culture (Sousa, Ardo and McSweeney 2001). Extent of proteolysis is highly influenced by several factors such as amount of coagulant, quality of milk, and types of starter culture.

4.4.4.1. Ripening index

Ripening index (soluble nitrogen/total nitrogen (TN) x 100) increased 7.28 to 32.25. % and 8.74 to 31.13 % in A2 and A1 Cheddar cheese respectively during ripening period up to 9 months (Fig 4.16). Secondary proteolysis, expressed as the 12% TCA SN/TN, increased 2.19 to 16.12 and 3.40 to 16.57 % in A2 and A1 Cheddar cheese during ripening period up to 9 months.

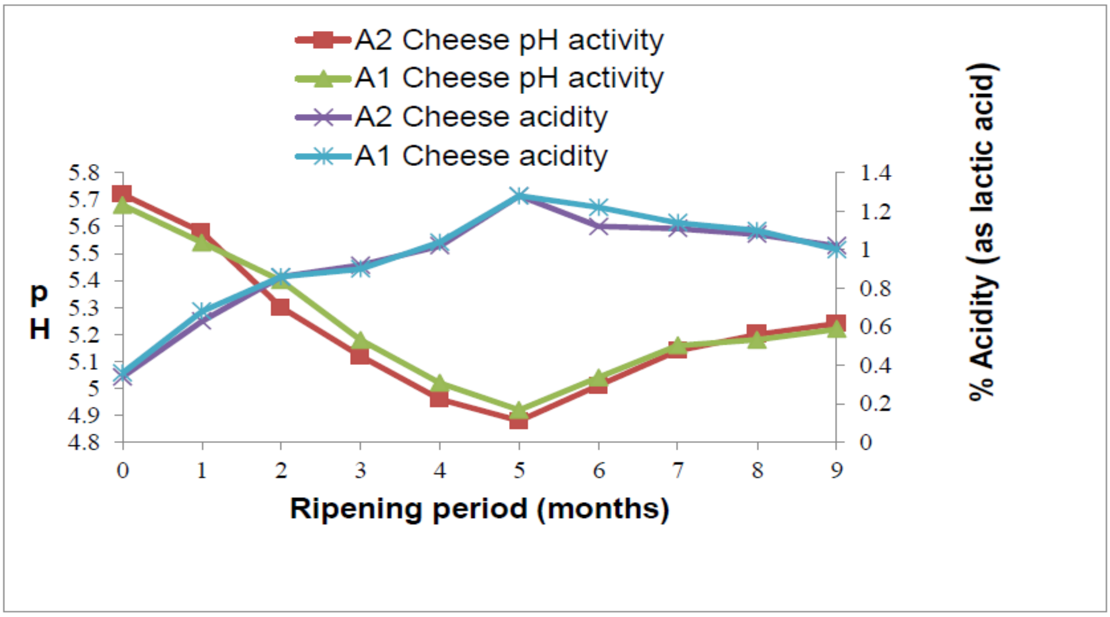


Fig. 4.16. Degree of proteolysis in terms of ripening index during ripening of A1 and A2 Cheddar cheese

The increased in primary and secondary proteolysis was due to significant complex biochemical changes during ripening process. Increased in soluble nitrogen compounds during ripening showed the hydrolysis of casein by the action of chymosin, milk proteases and starter cultures present at the initiation of ripening (Irigoyen et al., 2001). Excessive proteolysis observed in both type of Cheddar cheeses after four months of ripening, there was no significant difference observed between A1 and A2 cheese. 12% TCA SN/TN increased during ripening up to 9 months, but at a lower rate as compare to primary proteolysis (Fig 4.16). This fraction indicates the amount of small peptides and amino acids released by microbial proteinases and peptidases, mainly due to activity of starter and non-starter lactic acid bacteria and their enzymes (Sousa, Ardo and McSweeney 2001). This secondary proteolysis is an important biochemical activity for flavour development in cheeses. Amino acids released during proteolysis act as precursors for a range of catabolic bio chemical reactions which produce many important volatile flavouring compounds. Our findings were in accordance with Mendia*et al*. (2000). Researcher reported that proteolysis increased markedly with ripening time and highest levels of proteolysis being attained at the end of the ripening period i.e. after 6 months. They also mentioned that soluble nitrogen and free amino acid residues also increased significantly during ripening ($p>0.05$). Rynne *et al*. (2004) also reported the similar findings that primary proteolysis measured in terms of soluble nitrogen increased from 2.8% to 27.9% during ripening period of 1 year in half fat Cheddar cheese along with significant increased in secondary proteolysis.

4.4.5. Identification and quantification of BCM 7 and BCM 7 like peptides during ripening and simulated *in-vitro* digestion of Cheddar cheese prepared by A1 and A2 milk

To study the effect of ripening on release of BCM 7 and BCM 7 like peptides in Cheddar cheese was evaluated up to 9 months of maturation at 3-4^{0}C (Table 4.14). BCM 7 and BCM 7 like peptides were not released in fresh Cheddar cheese prepared from A1 and A2 milk (Fig 4.20 a).

Table 4.14. Identification and quantification of BCM 7 and BCM 7 like peptides during ripening and simulated *in-vitro* digestion of A1 and A2 Cheddar cheese

Storage Period (Months)	Water solubleextract (µg/g)		Salivary extract (µg/g)		Gastric extract (µg/g)	Intestinal extract (µg/g)		
	A2	**A1**	**A2**	**A1**	**A2**	**A1**	**A2**	**A1**
Fresh	nd	Nd	nd	nd	nd	nd	6.72±2.40	71.02±18.24
1	nd	Nd	nd	nd	nd	1.24±0.24	6.19±0.06	63.86±8.60
2	nd	Nd	nd	nd	0.76±0.342	1.11±0.45	4.84±1.96	44.51±10.12
3	nd	Nd	nd	nd	0.62±0.02	1.40±0.76	5.56±1.16	38.98±18.06
4	nd	0.19±0.08	nd	0.19±0.01	0.58±0.12	1.17±0.60	4.40±2.24	21.0±3.40
5	nd	0.11±0.06	nd	0.20±0.08	0.64±0.06	5.96±0.34	4.48±0.72	16.84±2.68
6	nd	0.23±0.11	nd	0.36±0.16	1.54±0.06	5.32±0.06	5.02±4.60	16.46±3.34
7	0.05±0.03	0.16±0.06	0.10±0.06	0.48±0.12	1.62±0.246	5.56±0.36	3.03±0.16	5.56±0.15
8	0.03±0.03	0.26±0.24	0.04±0.08	0.22±0.08	1.56±0.36	2.46±0.32	4.28±1.0	9.48±0.44
9	0.31±0.01	0.075±0.03	0.24±0.12	0.63±0.03	1.88±0.18	2.40±1.6	3.09±1.2	4.15±0.06

BCM 7 and BCM 7 like peptides were not released in fresh Cheddar cheese prepared from A2 and A1 milk. Even up to 3 months of storage release of BCMs did not observed in both types of cheeses. These results indicate that BCM 7 like peptide may not be probably formed from β-CNs or present in very low concentration unable to quantify by RP-HPLC method. Muehlenkamp and Warthesen (1996); Alli *et al*. (1998) also did not detect BCM 7 or other BCMs in Brie and Cheddar cheese.

Presence of BCM 7 and BCM 7 like peptides was observed in A1 cheese only after 4 months of ripening period (Fig 4.20 b). Up to 9 months these peptides were identified in A1 cheese and concentration of such peptides were in the range of 0.075-0.26 μg/g. surprisingly presence of BCM 7 like peptides was also identified in A2 Cheddar cheese after 7 months of ripening time. Concentration of such peptides in A2 cheese was in the range of 0.03-0.31 μg/g. There was no specific trend observed between ripening time and concentration of BCM 7 and BCM 7 like peptides in both types of Cheddar cheese. These findings may be due to release of BCMs and simultaneously further degradation to smaller fragments as proteolytic enzymes of microbial origin can generate opioid peptides in dairy products but the released peptides may also be further hydrolysed to di or tri peptides by other bacterial enzymes such as e.g., by Xprolyl-dipeptidyl- aminopeptidase (PepX) enzymes of LAB (Donkor *et al*., 2007b). De Noni *et al*. (2015) also isolated about 0.27 mg/kg of BCM 7 in undigested commercial Cheddar cheese. Authors also identified BCM 7 in the range of 0.12 -0.14 mg/kg in Maasdam and Grana Padano cheeses, where as highest amount of BCM 7 was identified in Gorgonzola cheese (7.63 mg/kg). Release of peptides during ripening is influence by environmental factors such as pH and salt concentration of the cheese up to great extent (Muehlenkamp and Warthesen, 1996). β-CN derived water soluble peptides were isolated from Cheddar cheese by Muehlenkamp and Warthesen (1996). Haileselassie *et al*. (1999) also isolated BCM 7 in emulsified Cheddar cheese after an eight hour of incubation. However, generated BCM 7 was degraded by bacterial and fungal peptidases within 72 hour incubation. Therefore, BCM 7 may be generated from hydrolysis of β-CNs during cheese ripening, simultaneously degrade into smaller peptides by LAB during ripening process.

Majority of these peptides released from the centre of β-CN molecule especially from N- terminal cleavage i.e. β-CN (53-91) (Souca, Ardo and McSweeney 2001). Peculiarity of this fragment is that it contains encrypted BCM 7 and alike peptides. There may be a strong probability of generation of BCMs in Cheddar cheese during ripening and enzymatic hydrolysis.

Sienkiewicz-Szłapka *et al*. (2009) also identified BCM 7 in hard and semi hard varieties of cheeses. Authors reported that mould ripened cheeses contained higher amount of BCMs in the range of 166–648 mg/100 g than the semi-hard bacterial ripened cheeses that contained lower amount of BCMs (4–100 mg/100 g). A number of studies reported the presence of BCM 7 and Pro BCMs in various types of cheeses, e.g., Cheddar, brie, kaszkawal, camping, swiss, elsberg, gouda, feta, blue, parmesan and crescenda (Smacchi and Gobbetti 1998; Jarmolowska *et al*., 1999; Jinsma and Yoshikawa 1999, Saito *et al*, 2000; Norris *et al*., 2003; Toelstede and Hofmann 2008; Sienkiewicz- Szłapka *et al*., 2009; De Noni *et al*., 2015). De Noni and Cattaneo (2010) also identified BCM 7 in commercial Cheddar cheese and concentration was 0.11mg/ kg. Norris *et al*. (2003) reported that optimum ripened market Cheddar cheese consist of 0.979 μg/g of BCM 7.

Most of these studies were carried out in commercial cheese samples in which β-CN variant in products were not known. These cheese samples might be prepared from mixing of milk with varying β-CN variant. So it is difficult to compare our findings with literature available till date.

The most probable release of BCM 7 and BCMs in milk products are during enzymatic hydrolysis or digestion (Jinsmaa & Yoshikawa, 1999; Cieślińska *et al*., 2012). A number of study identified pro BCMs in Cheddar and other variety of cheeses (Stepaniak *et al*., 1995; Smacchi & Gobbetti, 1998; Saito *et al*., 2000; Toelstede and Hofmann 2008). Presence of pro BCMs in undigested samples may be further hydrolysed to BCM 7 during ripening or digestion (Jinsmaa and Yoshikawa 1999). Even in our previous studies with processed milk and *dahi* (Section 4.2 and 4.3) release of BCM 7 and BCM 7 like peptides were seen only after the simulated digestion. MS data of our previous study as described in (Sections 4.2. and 4.3) provide the evidence that number of BCM 7 like peptides generated along with BCM 7 peptide during digestion of milk and milk products. So it is better to name as BCM 7like peptides instead of BCM 7 during interpreting RP-HPLC results. Very few studies were carried out to study the occurrence of BCMs during digestion. Therefore Cheddar cheese containing A2 and A1 variants of β-CN, were assessed for release of BCM 7 like peptides during digestion at different stages of ripening. Accordingly, Cheddar cheese samples were digested using simulated gastro intestinal digestive protocol (Minekus *et al*., 2014).

After salivary phase similar results were obtained as reported above for undigested Cheddar cheese samples. As there was no proteolysis in oral phase so BCMs were not reported in salivary phase of Cheddar cheese prepared from A1 and A2 milk (Table 4.14).

During gastric digestion there was no BCMs observed in fresh A2 and A1 Cheddar cheese but after 1 month of ripening BCM 7 like peptides were identified after gastric digestion of A1 Cheddar cheese (Table 4.14) (Fig. 4.20 c). Whereas in gastric extracts of A2 Cheddar cheese presence of BCM 7 like peptides were seen after 2 months of ripening. Concentrations were higher in gastric extracts of A1 Cheddar cheese compare to A2 Cheddar cheese. These observed differences certainly arose from variation in β- CN breakdown depending upon the different action of indigenous enzymes especially plasmin and other proteinases and peptidases in A1 and A2 Cheddar cheese. It is very difficult to have a consistent concentration of these enzymes in Cheddar cheese, because concentration of milk enzymes varying in animal to animal. There was no significant variation in BCM 7 likes peptides in gastric extracts of A2 Cheddar cheese throughout the ripening period and concentration varied between 0.58-1.88 μg/g.

Contrary to these findings BCM 7 decreased during ripening in gastric extracts of A1 Cheddar cheese. Initially concentration of BCM 7 in gastric extracts of A1 Cheddar cheese was 6.72 μg/g and it reduced to 3.09 μg/g after the nine months of ripening. About 50% reduction of BCM 7 like peptides observed during gastric digestion on completion of 9 months of ripening period. De Noni *et al*., (2015) also identified BCM 7 in gastric extracts of Cheddar cheese (0.25 mg/kg) along with other peptides such as BCM 4 peptide.

After simulated *in-vitro* gastrointestinal digestion, BCM 7 like peptides were reported in all intestinal extracts of A2 and A1 Cheddar cheese (Table 4.14 and Fig. 4.20 d). The releases of BCM 7 like peptides were approximately 10 times higher in A1 than A2 Cheddar cheese (Fig 4.17). On comparison with undigested and gastric extracts it was observed that concentration of BCM 7 like peptides were higher in intestinal extracts irrespective to ripening time and variants of β-CN present in Cheddar cheese. De Noni and Cattaneo (2010) also reported that after *in-vitro* gastrointestinal digestion amount of BCM 7 significantly increased compared to the undigested samples. Similarly De Noni *et al*. (2015) also reported that after *in-vitro* gastrointestinal digestion of commercial Cheddar cheese amount of BCM 7 increased about 5-folds compared to the gastric digestion. Higher concentration of BCM 7 like peptides in A1 Cheddar cheese is related to β-CN sequence specific to amino acid residue 'His' at position 67th position. The peptide bond between Ile_{66}-His_{67} in A1 variant is readily hydrolysed by gastrointestinal enzymes than the peptide bond between Ile_{66}-Pro_{67} in A2 variant. Jinsmaa and Yoshikawa (1999) and Ul Haq *et al*. (2015) reported that under normal digestive condition A2 β-CN releases much less or no BCM 7.

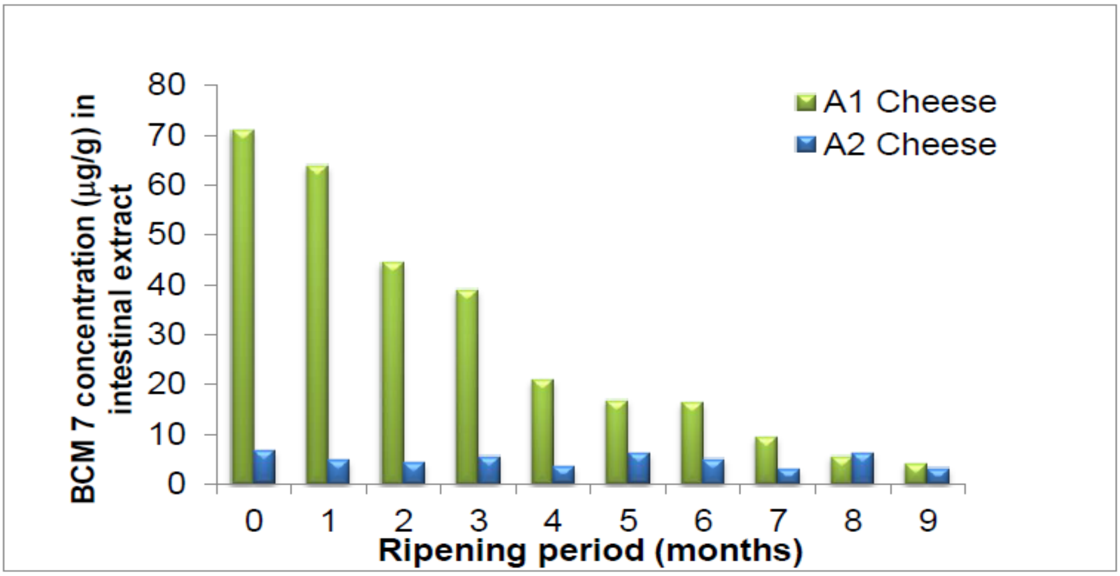


Fig 4.17. Effect of ripening on BCM 7 and BCM 7 like peptides in simulated gastrointestinal extracts of A1 and A2 Cheddar cheese during ripening using RP- HPLC method

To evaluate the effect of ripening on release or degradation of BCM 7 in Cheddar cheese, study was carried out up to 9 months (Fig 4.17). It was observed that concentration of BCM 7 decreased as ripening processed especially in intestinal extracts. During initial stage of ripening concentration of BCM 7 like peptides were in range of 4.68 to 10.58 μg/g in A2 Cheddar cheese and 60.56-72.02 μg/g in A1 Cheddar cheese. Reduction in BCM 7 like peptides was not significant in A2 Cheddar cheese being low content of the peptides. As far as A1 Cheddar cheese concern changes in BCM 7 was significant with ripening period. In our study it was observed that reduction of BCM 7 like peptides were more significant after 3 months of ripening with a 50% reduction of these peptides. After 9 months of ripening concentration were 3.09 ± 0.01μg/g and 4.15 ± 0.06 μg/g in A2 and A1 Cheddar cheese respectively (Table 4.14).It was observed that after completion of ripening of 9 months concentration of BCM 7 like peptides in both Cheddar cheeses were nearly same. Although there was significant reduction of BCM 7 like peptides was observed in A1 Cheddar cheese. Release of water soluble peptides in cheese and in their *in-vitro* digestive extracts depends on the number of factors such as quality of rennet, quality of milk, microbial culture, pH, acidity, salt concentration etc. In the preparation of Cheddar cheese ripening is the main step to get a characteristic product with unique flavour. During ripening a complex biochemical changes takes place mainly in proteins. Extensive proteolysis leads to generation of unique flavour due to release of number of water soluble peptides. These water soluble peptides formed mainly from 53-91 fragments of β-CN (Sousa, Ardo and McSweeney 2001). So release of more and more water soluble peptides may leads to formation of BCM 7 like peptides. De Noni and Cattaneo (2010) also reported that Cheddar cheese showed highest release of BCM 7 content in the range of 15.22 mg/kg after *in-vitro* intestinal digestion compared to other cheese varieties. Reduced concentration of BCM 7 like peptides may be due to lactic acid bacterial activity as ripening proceed microbial activity increased. Lactic acid bacteria posses' Xprolyl-dipeptidyl-aminopeptidase (PepX) activity may further hydrolysed BCM 7 to di or tri peptides (Donkor *et al*., 2007b). Muehlenkamp and Warthesen (1996) also reported that proteolytic system of *L. lactis* ssp. *cremoris* can degrade BCM 7 to half of its original concentration within 6–15 weeks of storage. It was also observed in present study that rate of secondary proteolysis increased with ripening and extent of proteolysis was more after the 4 months of ripening. Secondary proteolysis indicates the small peptides (amino acid residue 2-20) hence it can be correlated that as proteolysis increases degradation of BCM 7 also increases. Our findings were also supported by study conducted by De Noni and Cattaneo (2010) BCM 7 was not observed in 35-day stored taleggio cheese prepared using lactic acid bacterial strains of *L. delbrueckii* ssp*. bulgaricus* and *S. thermophilus* as the starter culture.

4.4.6. Quantification and detection of BCM 7 in Cheddar cheese during ripening prepared from A1 and A2 milk by ELISA assay

Quantification of BCM 7 in Cheddar cheese prepared from A1 and A2 milk by ELISA assay

ELISA technique was used to cross validate the results obtained by RP-HPLC method for quantification of BCM 7 in simulated gastrointestinal digestive extracts of A1 and A2 Cheddar cheese (Fig 4.18 and 4.19). Similar trend of release of BCM 7 was observed by ELISA assay also. Concentration of BCM 7 in gastrointestinal extracts of both types of Cheddar cheese obtained by ELISA assay were significantly lower than the concentration evaluated by RP-HPLC method in same products. Difference in the concentration of BCM 7 in both methods was due to presence of number of BCM 7 like peptides in digestive extracts which may have similar physicochemical and absorbance characteristics. Thereby providing more absorbance in RP-HPLC method so higher concentration of BCM 7 likes peptides. However, ELISA assay is specific to BCM 7 peptide and least or no interference by other similar peptides.

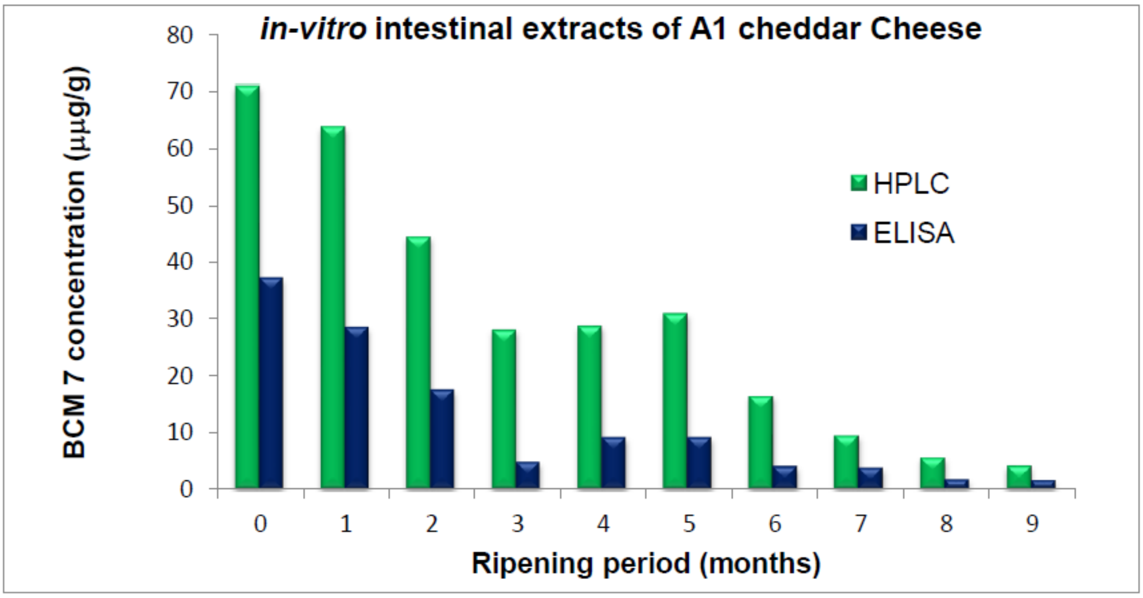


Fig. 4.18. Comparison of HPLC and ELISA assay for quantification of BCM 7 in intestinal extracts of A1 Cheddar cheese during ripening

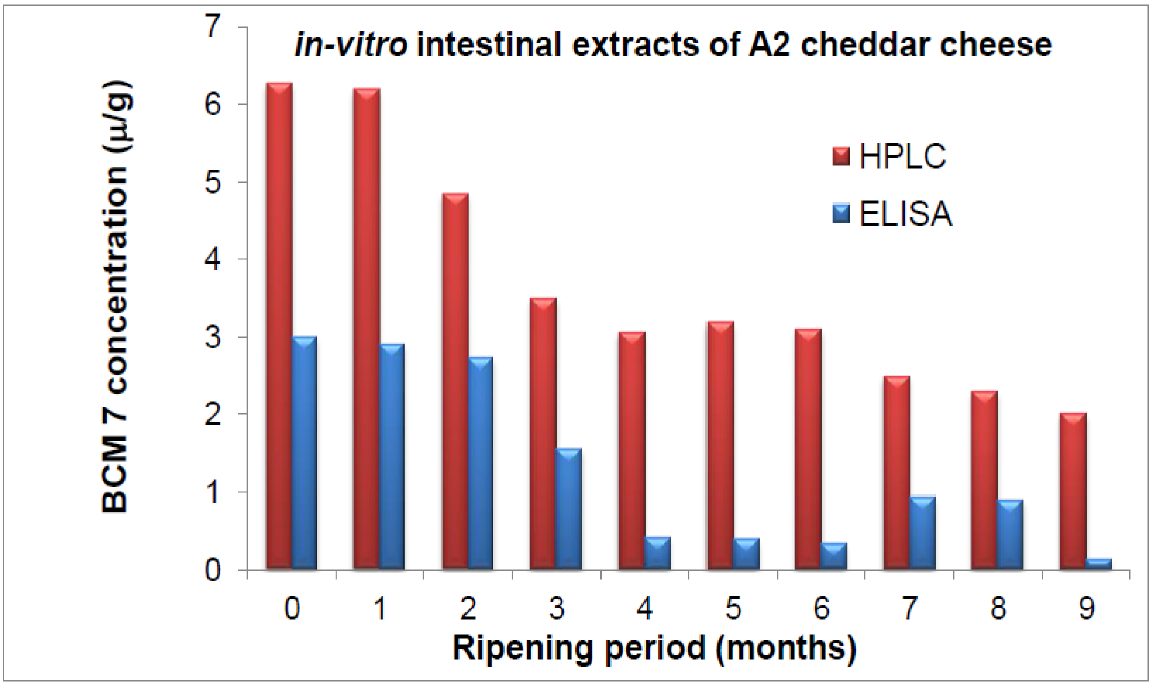


Fig. 4.19. Comparison of HPLC and ELISA assay for quantification of BCM 7 in intestinal extracts of A2 Cheddar cheese during ripening

To study the effect of ripening process on release of BCM 7 like peptides using ELISA assay, it was observed that release of BCM 7 peptide decreased with advancement of ripening on gastrointestinal digestion in both Cheddar cheeses. Finding showed that concentration of BCM 7 like peptides decreased as ripening proceeds (Fig 4.18 and 4.19). Very little data are available in literature related to the digestion of cheese and release of BCMs, so it is difficult to compare findings of our study. But in-vitro studied in other fermented dairy products provides the evidence that concentration of BCM 7 decreased on storage (Jarmołowska and Krawczuk, 2012). The decrease in content of BMC 7 during storage of fermented dairy products is due to PepX activity of lactic acid bacteria. BCM 7 and BCMs are proline rich peptide more resistant to digestive enzymesbut highly susceptible to LAB derived PepX enzymes that preferentially cleave peptide bonds involving proline amino acid residue. During storage PepX is present in the cytoplasm of microbial cells releases into the surrounding medium on autolysis of bacteria cells and PepX activity increased with storage time (Atlan *et al*., 1990). At low pH and low temperature storage condition of fermented dairy products, bacterial cells are more prone to lysis and release more PepX (Otte *et al*., 2011). Nielsen *et al*., (2009) also reported that exo proteases of lactic acid bacteria hydrolysed caseins into larger peptides, and further hydrolysis takes place in to di and tri-peptides by endoppetidases. Consequently, LAB may able to cleave β-CNs into BCM 7 and other related BCMs during fermentation and subsequently degrade them into even smaller peptides during storage. The finding reported by Kunda *et al*., (2012) supports the degradation theory of BCM 7 peptide. Authors were able to isolate a number of fragmented peptides i.e. β- CNs f (60-61) and β-CNs f (62-63) along with pro BCMs in yoghurt samples. These findings indicate that pro BCMs and BCM 7 can further hydrolysed to smaller peptides. Paul and Somkuti (2009) also reported that, fermentation with *L. delbrueckii* ssp. *bulgaricus* causes significant hydrolyses of antimicrobial and hypertensive peptides after 4 h incubation at low ie pH 4.5. This study suggests that any BCMs generated during fermentation may be further degraded on low temperature storage at low pH condition. Several studies reported that proteases and peptidases of LAB remained active throughout fermentation and post-storage in fermented dairy products. Recently Nguyen *et al*. (2018) studied the effect of fermentation on BCM 7 in UHT milk.

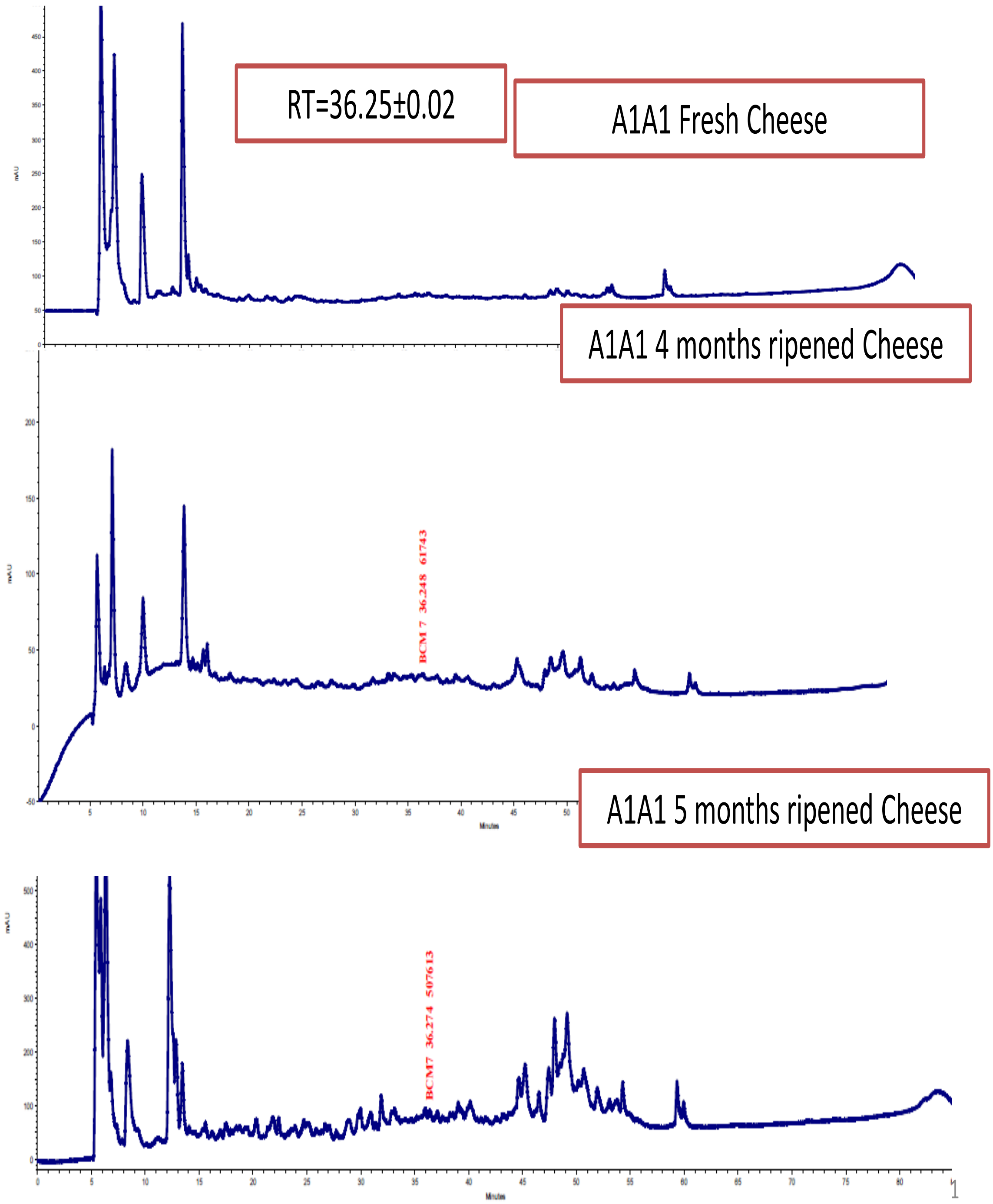


Fig. 4.20 (a) RP-HPLC chromatogram of A1 Cheddar cheese showing release ofBCM 7 during ripening

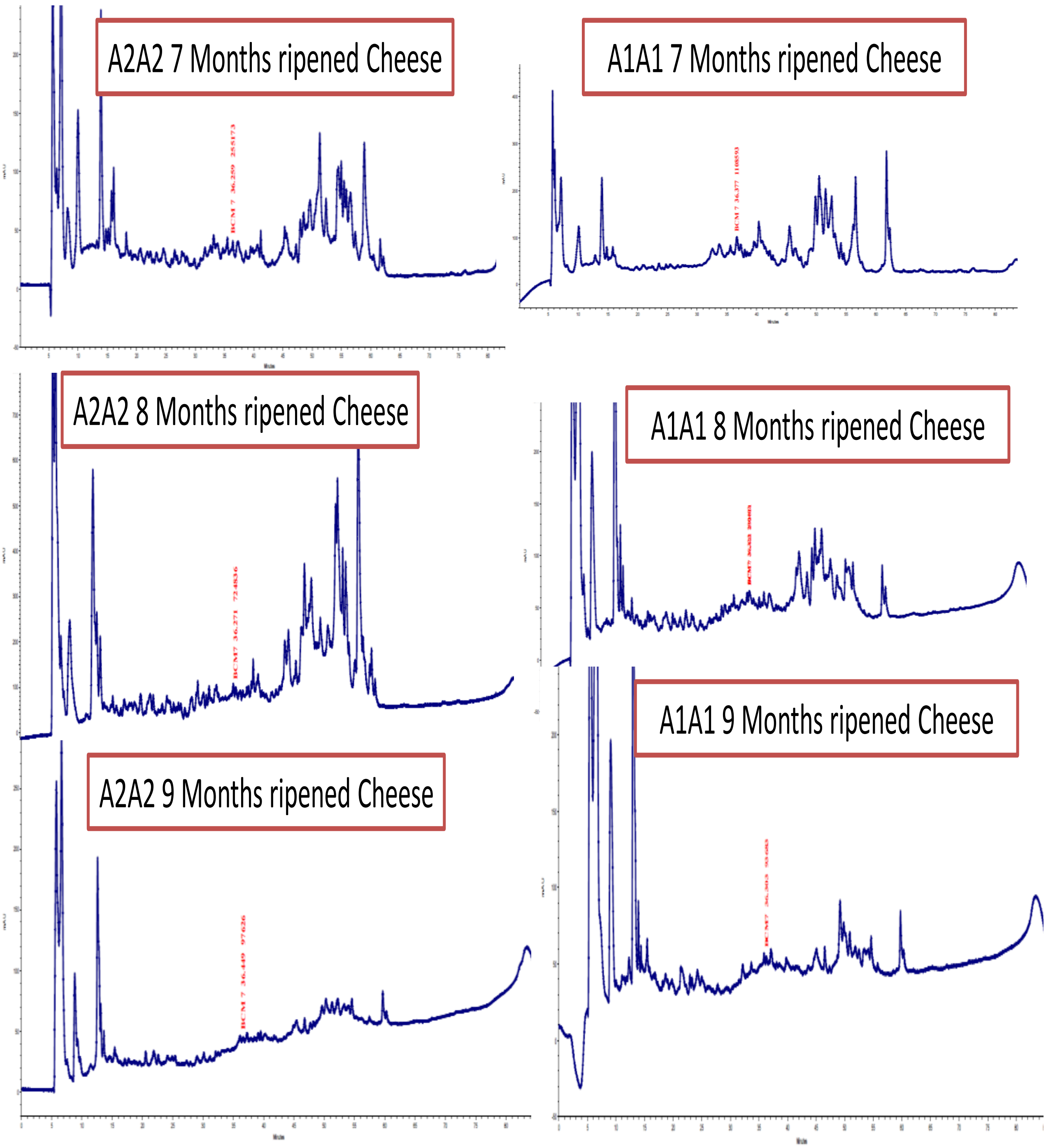


Fig 4.20 (b). Comparison of RP-HPLC chromatogram of A1 and A2 Cheddar cheese showing release of BCM 7 during ripening at different time

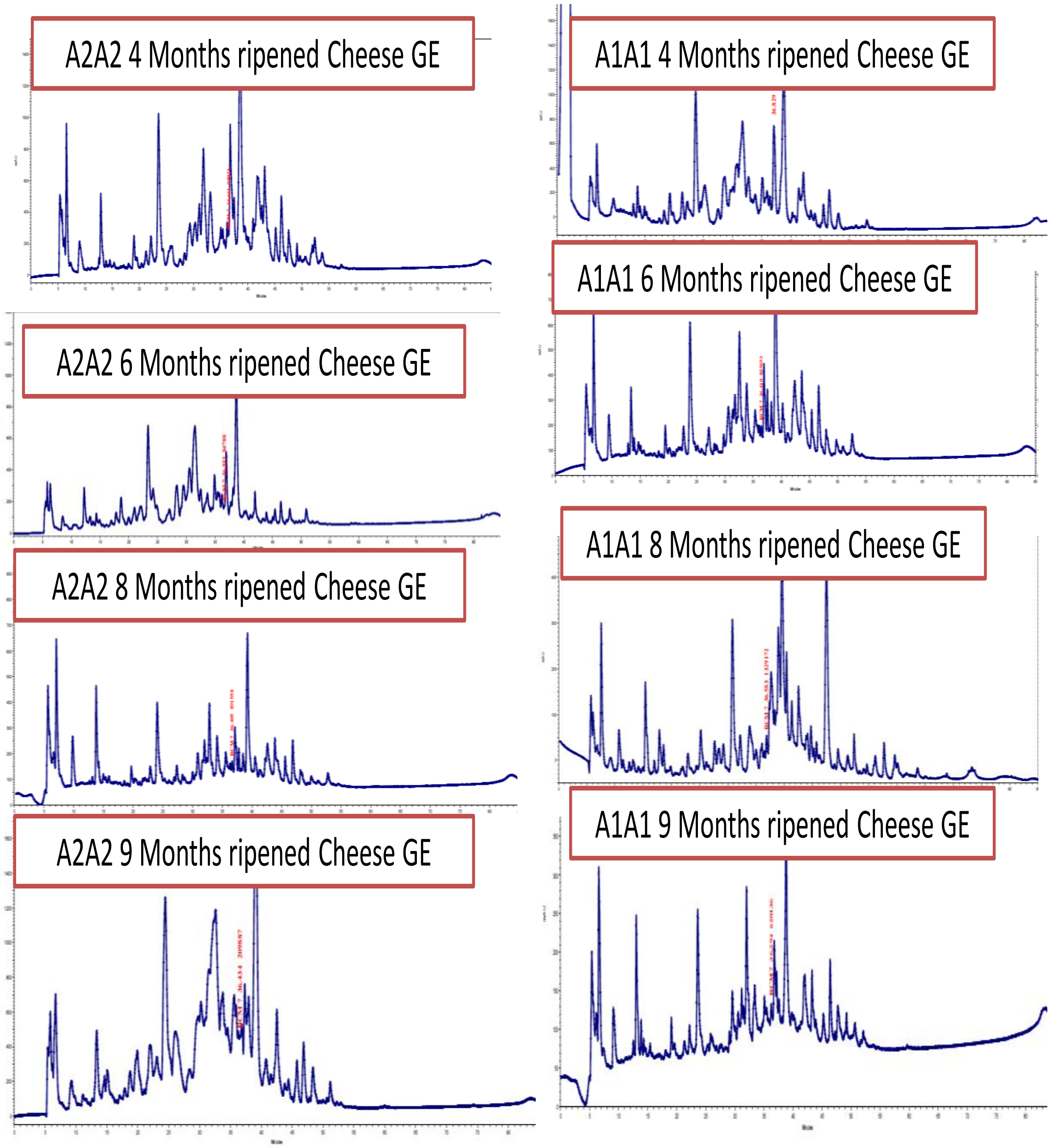


Fig 4.20. (c). Comparison of RP-HPLC chromatogram of A1 and A2 Cheddar cheese showing release of BCM 7 during ripening at different time (GE-Gastric Extract)

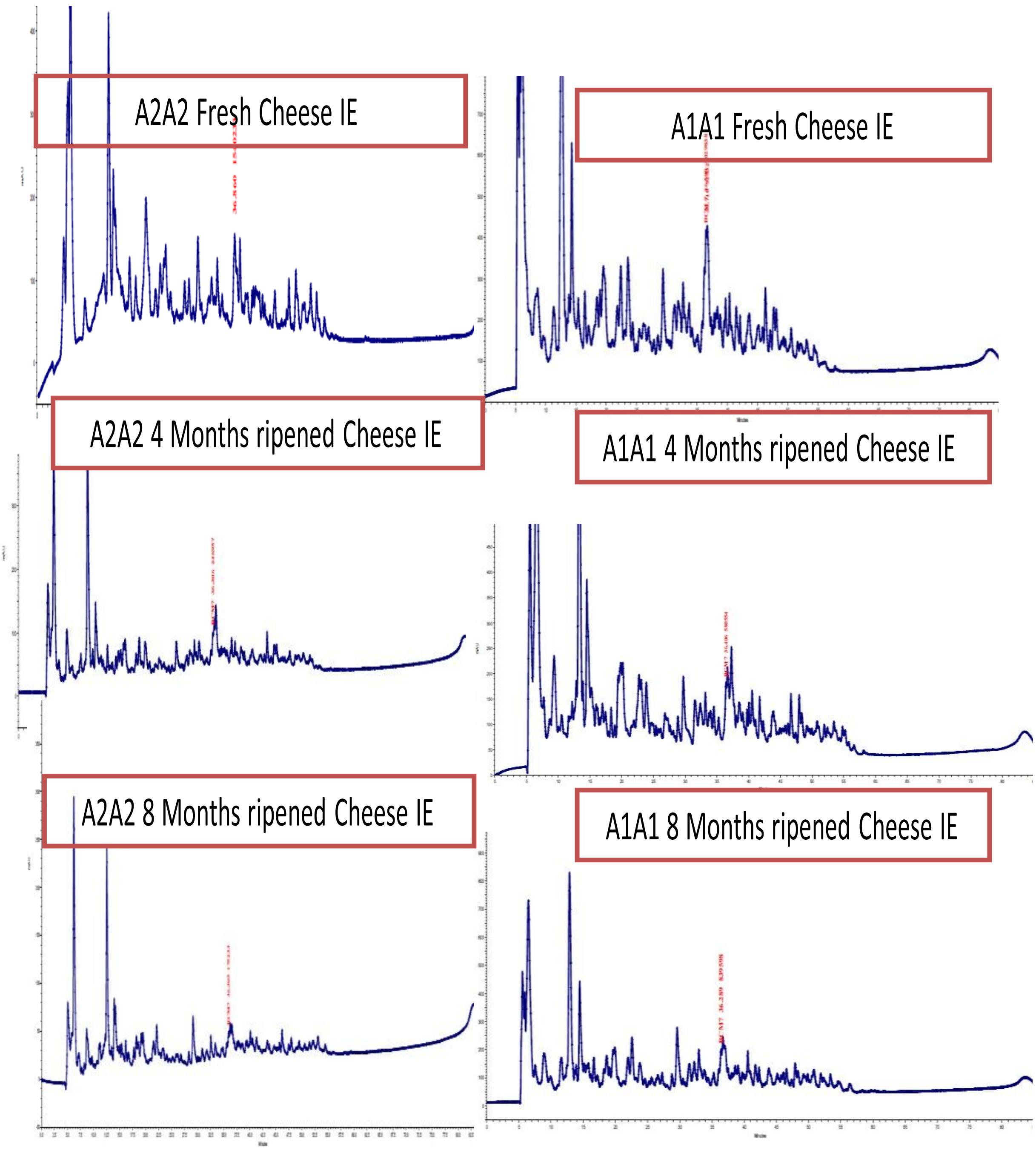


Fig 4.20. (d). Comparison of RP-HPLC chromatogram of A1 and A2 Cheddar cheese showing release of BCM 7 during ripening at different time (IE-Intestinal Extract)

Sample was spiked with BCM 7 peptide and fermentation was carried out using *Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus* bacteria. The findings revealed that lactic acid bacteria used in study were able to degrade entire added peptide at low pH condition. Hence this evidence suggested that lactic acid bacteria have ability to degrade BCM 7 peptide in fermented products. Reduction in BCM 7 concentration of both types of Cheddar cheeses strongly associated with increase in PepX activity during ripening period.

4.5. Evaluation of effect of acid coagulation on Release of BCM 7 and BCM 7 like peptides in Indian dairy product: *Chhana*

Chhana is a popular Indian dairy product prepared by acid coagulation of boiled hot cowwhole milk and subsequent drainage of whey. It is also known as *paneer* or Indian cottage cheese. The acids commonly used are lactic or citric, in both natural and chemical forms. It is generally used in the preparation of sweet meet. More technically *Chhana* is a heat induced acid coagulated milk gel mainly composed of a casein matrix associated with denatured whey protein, with embedded fat globules and water.

4.5.1. Preparation of *Chhana* from A1 and A2 milk

Chhana was prepared with A1 and A2 milk using standard protocol as shown in Fig(3.7)

4.5.2. Physicochemical analysis of fresh *Chhana* prepared from A1 and A2 milk *Chhana*

Channa prepared with A2 and A1 milk was evaluated for their proximate composition as shown in Table 4.17. There was no significant difference observed in proximate composition of both types of *Chhana* ($p<0.05$). Similar results were reported by Begum*et al*., (2019) and Singh and Kant (2013).

Table 4.17. Physicochemical analysis of fresh A1 and A2 *Chhana*

S.No.	Parameter	*A1 Chhana*	*A2 Chhana*
1	pH	5.46 ± 0.14	5.46 ± 0.12
2	% Moisture	57.86 ± 0.34	57.62 ± 0.48
3	%Protein	16.72 ± 0.12	16.54 ± 0.24
4	%Fat	18.24 ± 0.60	18.86 ± 0.32
5	%Ash	1.90 ± 0.12	1.86 ± 0.16

Values are mean ± SD, n=8

4.5.3. Effect of storage on the acidity of A1 and A2 *Chhana*

Chhana was stored for one week at refrigerated condition (4-5^{0}C). Acidity increased with storage period in both types of *Chhana* as shown in Fig. 4.21, but no significant difference observed between A1 and A2 *Chhana* ($p<0.05$). Similar results were reportedby Khatkar, Ray and Kaur (2017) for *paneer*.

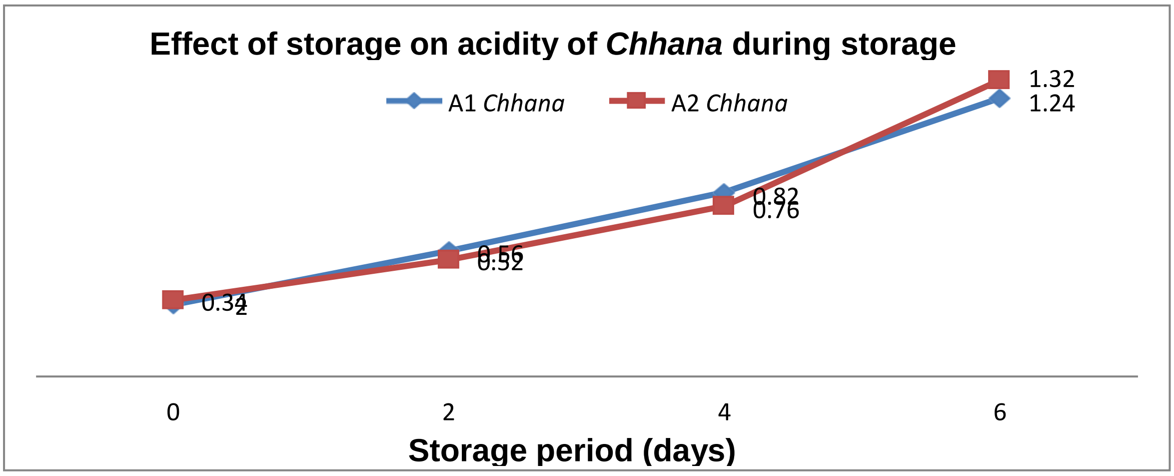


Fig 4.21. Determination of acidity as % lactic acid of A1 and A2 *Chhana* during storage at refrigerated temperature.

4.5.4. Determination of free fatty acid (FFA) of A1 and A2 *Chhana* during storage

To study the storage effect on stability of the product FFA was evaluated the index ofrancidity was carried out in both types of sample as depicted in Fig. 4.22. Duringstorage FFA content increased in both types of samples but there was no significantdifference observed between A1 and A2 *Chhana* ($p<0.05$).

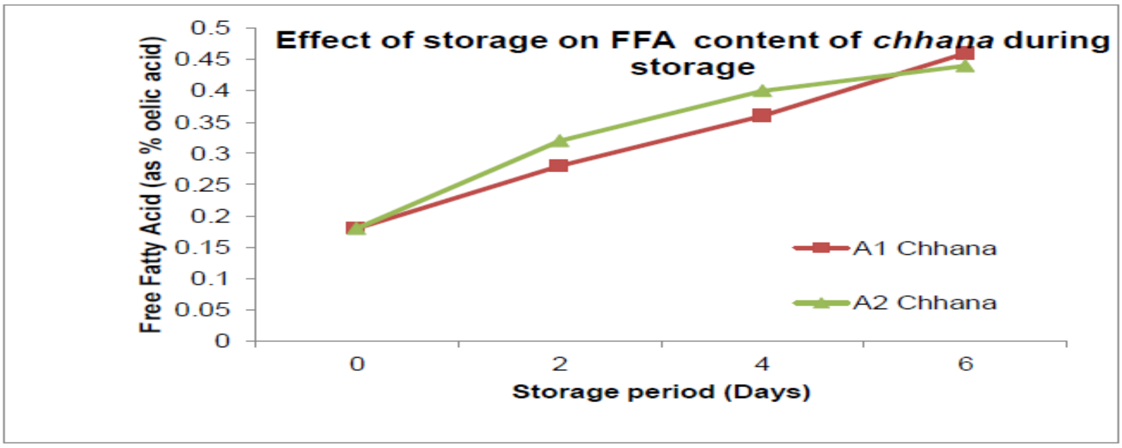


Fig. 4.22. Determination of free fatty acid (FFA) of A1 and A2 *Chhana* duringstorage at refrigerated temperature

4.5.5. Identification and quantification of BCM 7 in *Chhana* prepared from A1 and A2 milk

To evaluate the effect of coagulation on release of BCM 7, *Chhana* was prepared from A1 and A2 milk and analyzed for the occurrence of BCM 7 before and after gastrointestinal digestion using RP-HPLC method. BCM 7 and BCM 7 like peptides were not detected in *Chhana* prepared from A2 and A1 types of milk (Table 4.15).

Table 4.15. Quantification of BCM 7 in A1 and A2 *Chhana* and their gastrointestinal digestive extracts

Storage Period (Days)	Water soluble extract (μg/g)		Salivary extract (μg/g)		Gastric extract (μg/g)		Intestinal extract (μg/g)	
	A1	**A2**	**A1**	**A2**	**A1**	**A2**	**A1**	**A2**
Fresh	nd	nd	Nd	nd	nd	nd	8.36±0.56	3.34±0.12
2	nd	nd	Nd	nd	nd	nd	8.29±0.62	2.80±0.80
4	nd	nd	Nd	nd	nd	nd	4.84±0.12	1.83±0.24
6	nd	nd	Nd	nd	nd	nd	1.56±0.28	0.48±0.16

(Values mean ± SD, n=8)

These findings provide the evidence that release of BCM 7 requires a set of enzymes. Jinsmaa and Yoshikawa (1999) and Considine (1999) showed that the release of BCMs requires series of proteolytic enzymes such as pepsin, trypsin, and elastase. First pepsin cleaves the Leu_{58}-Val_{59} and in the presence of leucine amino peptidase (LAP), Val releases from N-terminus of BCM 7. No data is available related to the occurrence of BCM 7 in heat coagulated products so it is difficult to compare findings of the present study. In earlier section of present study showed that BCM 7 was not isolated from processed milk (Table 4.5) As expected simulated *in- vitro* digestive study of *Chhana* showed that 2 min oral digestion did not have any effect on milk proteins (Fig 4.23) and no BCM 7 and BCM 7 like peptides noticed in salivary extract of all samples. As there is no proteolytic enzyme in saliva hence no hydrolysis during oral phase (Benedé *et al*., 2014).

BCM 7 was not observed in the gastric extracts of *Chhana* prepared from A1 and A2 milk (Fig 4.24). Recently Lambers *et al*. (2021) and Nguyen *et al*. (2020) also reported that no pro BCMs or BCM 7 released were seen during gastric digestion of raw and heat processed milk. Similar findings were reported by Asledottir *et al*. (2017) and De Noni (2008). Even gastric digestion of purified β-casein fraction unable to generate BCM 7 (Ul Haq *et al*., 2015). As digestion of proteins molecules starts in the stomach and pepsin is only enzyme present in gastric juice acts on specific site in protein chain. Only partial hydrolysis occurs during gastric digestion. Jinsmaa and Yoshikawa (1999) and Considine (1999) very well explain the release of BCM 7 is multistep enzymatic process and required a set of enzymes such as pepsin, trypsin, elastase. Initially pepsin cleaved peptide bond between the Leu_{58}-Val_{59} and then trypsin and chymosin hydrolysed peptide bonds involving basic and aromatic amino acid residues respectively to form large molecular weight peptides. These peptides further hydrolysed to smaller peptides by amino peptidases, such as leucine amino peptidase removes Val from N- terminus of β-CN fragment and releases BCM 7. Pancreatic elastase is other enzyme involved in the generation of BCM 7 by relieving C-terminus amino acids by cleavage of Ile_{66}-His_{67} peptide bond in β- CN fragment (Jarmolowska *et al*., 1999). So release of BCM 7 did not occur after gastric digestion (Ul Haq *et al*., 2015; Jinsmaa and Yoshikawa 1999). Benedé *et al*. (2014) also reported that nearly 152 peptides with varying amino acid residues (3-21) released during digestion of β-casein by commercial porcine pepsin in simulated gastric and human fluid. Despite of release of number of small molecular weight peptides no BCM 7 was identified. Even yoghurt did not form BCM 7 after gastric digestion (Nguyen *et al*., 2020).

Contradictory to our findings some studies reported BCM 7 in gastric extracts of milkand milk products (Cieślińska *et al*., 2012 De Noni and Cattaneo 2010; De Noni *et al*, 2015).

Release of BCM 7 and BCM 7 like peptides were observed in all samples of *Chhana* prepared from A2 and A1milk after simulated *in-vitro* gastrointestinal digestion (Fig 4.25 and 4.26). Content of BCM 7 and BCM 7 like peptides in A1*Chhana* was 2.5-3.0 times higher than A2 *Chhana* (Table 4.18). Our findings were supported by number of studies conducted in milk samples.

Fiedorowicz *et al*. (2014) reported highest concentration of BCM 7 in A1 milk compare to other variants. It was also observed in same study that concentration of BCM 7 was 13-36 times higher in the enzymatic extract as compare to undigested milk. Asledottir *et al*. (2017 and 2018) and Cieślińska *et al*. (2007) also reported similar findings in raw milk. Present study suggested that higher concentration of BCM 7 and BCM 7 like peptides in A1 *Chhana* is related to specific amino acid substitution at 67th position in β-casein sequence. It is well reported in literature that under normal digestive condition A1 β-CN releases higher amount of BCM 7 than A2 (Jinsmaa and Yoshikawa 1999; Cieślińska at al., 2007; De Noni 2008; EFSA 2009; Ul Haq *et al*., 2015). Boutrou *et al*. (2013) also reported that pro BCMs and BCM 7 were most frequent proline rich peptides released after intestinal digestion of milk and milk products in healthy human. They also identified BCM 7 throughout the intestinal digestion in the human. Surprisingly peptide concentration decreases as time passes. The results obtained in our study supports the theory of A2 milk is more resistant to digestion consequences of this leads to less production of BCM 7. Contradictory to our findings, Recently Nguyen *et al*. (2021) also identified 127.25 to 198.10 ng/ml BCM 7 in β-CN A1 and 19.35 to 24.50 ng/ml in β-CN A2I but no BCM 7 released from β-CN A2 variant.

Chhana samples were stored at refrigerated condition for one week and analyzed to study the release of BCM 7 and BCM 7 like peptides. During storage also *Chhana* prepared from A1 milk showed highest concentration of BCM 7 like peptides than A2 *Chhana.* Content of BCM 7 like peptides decreased on storage up to 6 days of study.

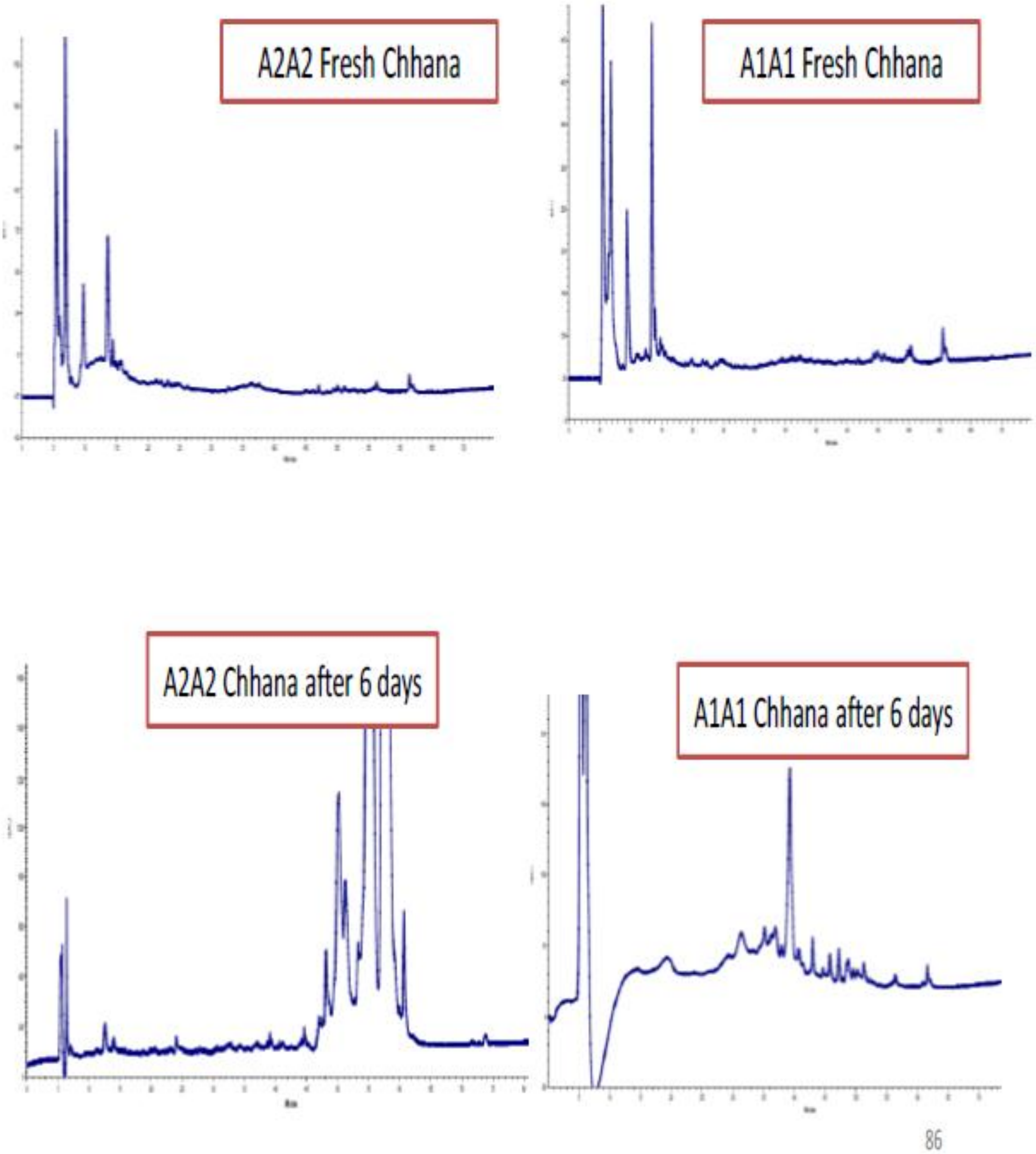


Fig. 4.23 . RP-HPLC chromatogram of A1 and A2 *Chhana* during storage

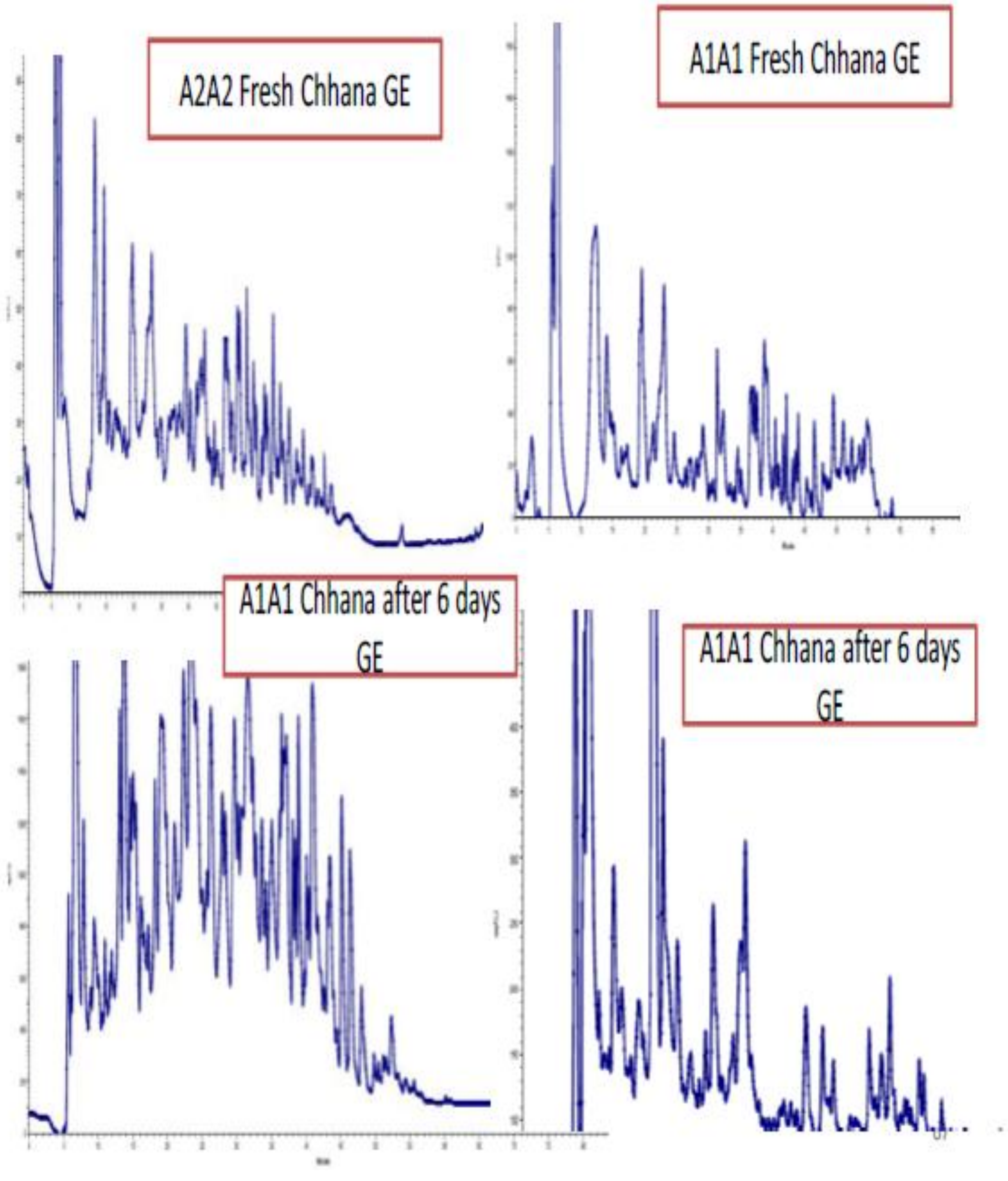


Fig. 4.24. RP-HPLC chromatogram of *Chhana* during storage (GE-Gastric Extract).

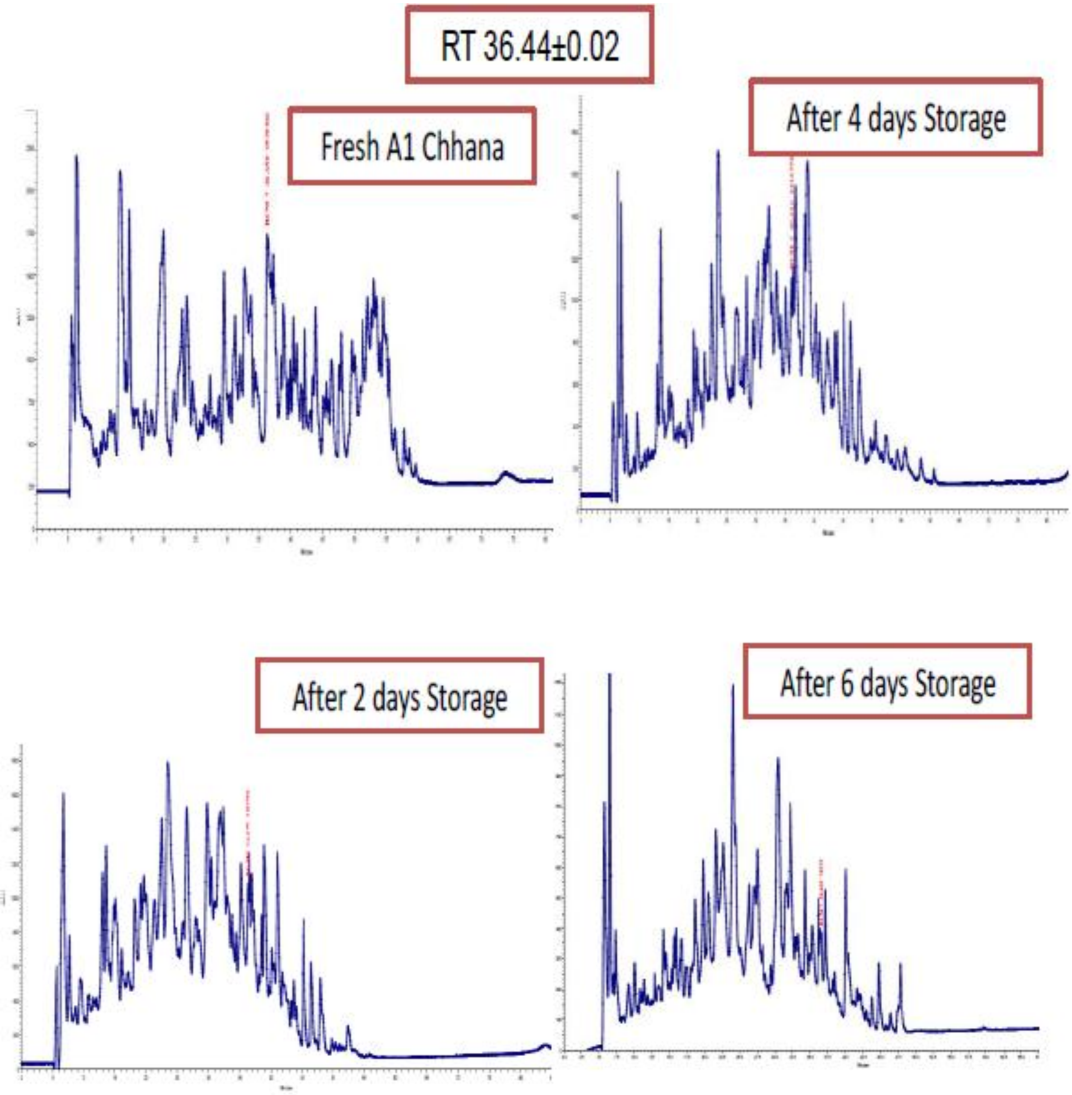


Fig. 4.25. RP-HPLC chromatogram of *Chhana* during storage (IE-Intestinal Extract).

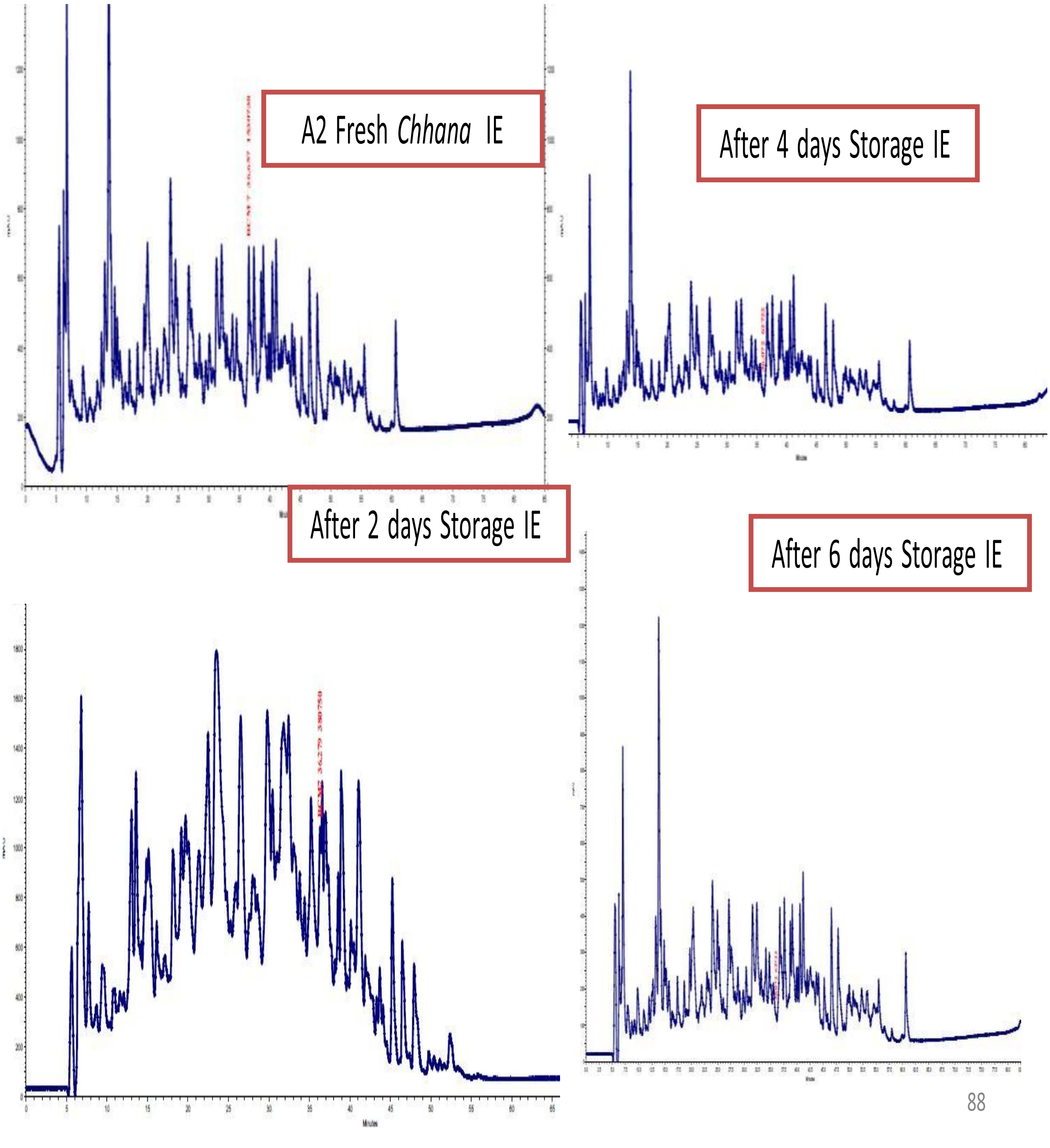


Fig. 4.26. RP-HPLC chromatogram of A2 *Chhana* during storage (IE-Intestinal Extract).

4.5.6. Quantification of BCM 7 in A1 and A2 *Chhana* by ELISA assay

Application of ELISA based assay for the quantification of BCM 7 in milk and milk products were successfully used by a number of researcher, being a highly sensitiveand easy to perform (Sienliewicz-Szłapka *et al*., 2009; Cieślińska *et al*., 2012). There by to cross check the results of RP-HPLC method, gastrointestinal digestive extracts of *Chhana* prepared from A1 and A2 milk were analyzed by ELISA assay (Fig 4.27 and 4.28). Identification and quantification of BCM 7 by ELISA assay also showed similar trend of occurrence of BCM 7 in all samples as identified by RP-HPLC method. Contradictory to other studies, surprisingly there was no significant difference in the concentration of BCM 7 and BCM 7 like peptides evaluated by both methods. This may be due to the least generation of BCM 7 like peptides in such types of coagulated product. Hence least interference of such peptides with quantification of BCM 7 compare to processed milk, cheese and *dahi*.

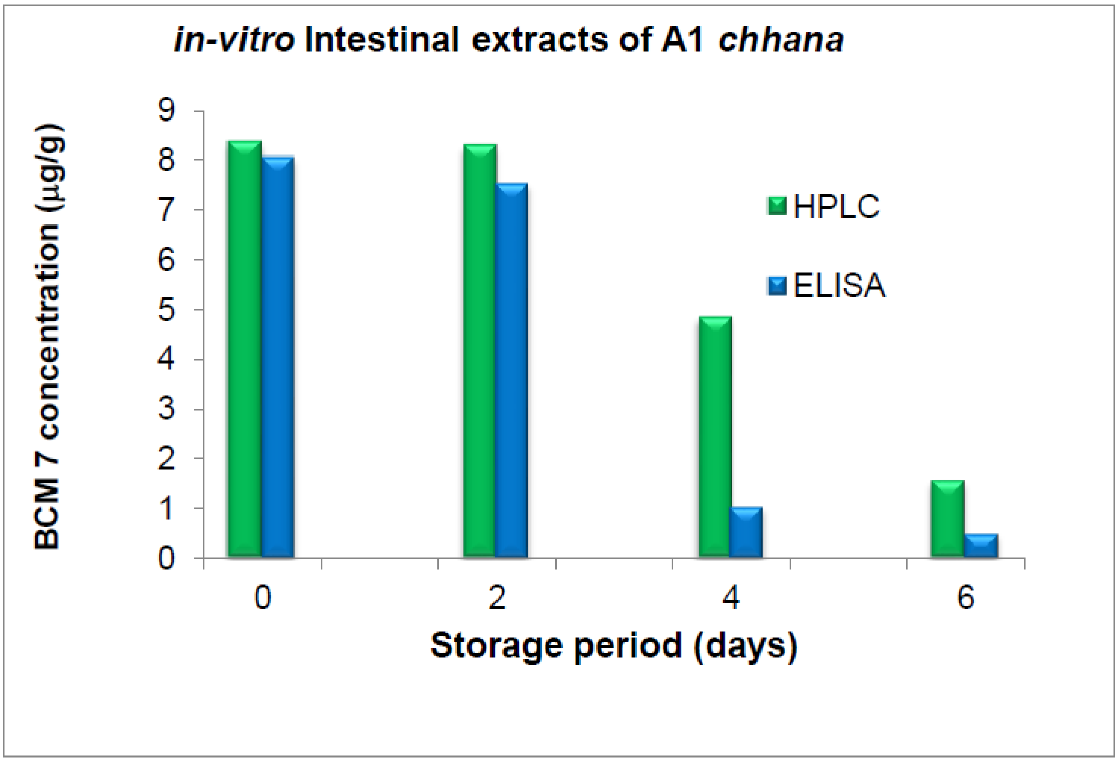


Fig 4.27. Comparison of RP-HPLC method with ELISA assay for quantification of BCM 7 in A1 *Chhana* during storage

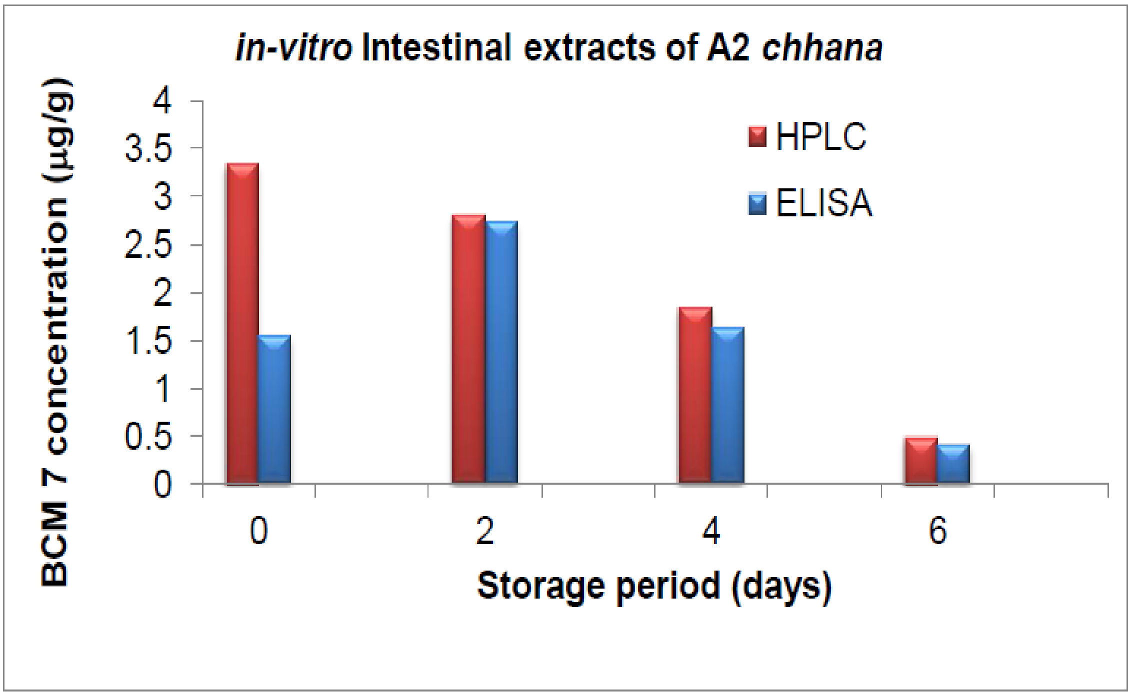


Fig 4.28. Comparison of RP-HPLC method with ELISA assay for quantification of BCM 7 in A2 *Chhana* during storage

CHAPTER –5

Summary and Conclusions

SUMMARY AND CONCLUSION

Beta casomorphin 7 (BCM 7) is the most potent opioid peptide seems to be responsible for the range of chronic diseases in human being. Their release is linked to the A1 variant of β-CN during dairy processing and identified in various milk and milk products. There are certain studies which provide the evidence that release of BCM 7 not only linked to milk containing A1 variant of β-CN but also identified in milk containingother variants of β-CN. So inline of above information this work was undertaken whetherBCM 7 does form at various processing steps or degrades requires further investigation. Therefore the aim of the study is to investigate the effect of different dairy processing condition on the formation and degradation of BCM 7.

In this study, an attempt has been made to standardize RP-HPLC coupled with UV- detector for quantification of BCM 7 in milk system for routine analysis. This successful validated method has been applied for the identification and quantification of the BCM 7 peptide in milk and milk products and their *in-vitro* digestive extracts to study the effect of different dairy processing condition on generation or degradation of BCM 7. The results obtained by RP-HPLC methods were cross checked by ELISA assay. Simultaneously peptide sequencing analysis was carried out using LCMS/MS for the authentication of BCM 7 in milk products by collecting the representative BCM 7 fractionfrom RP-HPLC analysis. Findings of the present work was summarized here as

- **RP-HPLC** method has been successfully standardized for quantification of BCM 7 in milk and milk products. The condition for separation of BCM 7 was optimized on ZORBAX 300SB-C18 LC column (4.6 x 250 mm x 5μm) by injecting20 μl of standard solutions of BCM 7 at lower level (0.25 μg/ml and 1.0 μg/ml) at flow rate of 0.5 ml/min using gradient run start with 90% solvent A (acetonitrile with 0.09% TFA) for 10 min, followed by gradual decrease in concentration of solvent A by 5% for every 10 min up to concentration of 70%. Then linear gradient was maintained for 30 min with solvent A and Solvent B (ultrapure water with 0.1% TFA) concentration (70:30). After that, concentration of solvent A was further decreased by 10 % with every 10 min till the ratio of solvent A and B reached to (10:90). The chromatograms were recorded at 220 nm for the study. The linear range was tested using calibration standard and Regression coefficient (R^2) for BCM 7 was 0.9884, indicating good linearity in the tested range. Instrument LOD and LOQ were 0.03 and 0.09 μg/ml respectively atretention time of 36.35 ± 0.10 min.

- For method validation, calibration standards were prepared in milk matrix. Regression coefficient (R^2) for validated method was 0.98, indicating good linearity in tested range. Method LOD and LOQ for BCM 7 were 6.06 and 20.205 μg/ml respectively with retention time of 36.44 ± 0.017 min.
- For evaluating accuracy and precision of standardized method in milk system, samples were spiked with 4 different known concentrations of BCM 7 and extraction of target analyte was carried out in triplicate. The accuracy of the method was 90.00-103.00 % with 1.2 to 7.8 % precision in terms of relative standard deviation.
- For assessing the reproducibility of standardized method, repeated measurements of BCM 7 in spiked milk at 4 different levels were carried out on three consecutive days. The relative standard variation of analytical method was 0.79 to 3.76 % indicating good inter-day reproducibility in tested range.
- To observe the effect of complex matrices on analysis of BCM 7 in milk system, matrix effects were calculated by comparing absorbance of BCM 7 in pure solvents to the milk system. The complex matrix of milk has significant effect on BCM 7. Milk spiked BCM 7 showed 35-45% lower response as compared to its aqueous solution.
- Proximate analysis of milk from A2A2 and A1A1 genotypes cow showed no significant difference in the chemical composition of both type of milk ($p>0.05$).
- In order to assess the relation between extent of proteolysis and occurrence of BCM 7, degree of hydrolysis was calculated in terms of mmol of leucine release per gram of protein. It was found that there was

no hydrolysis in oral phase because saliva did not contain any proteolytic enzyme. In gastric phase around 40-50 % proteolysis occur in 2 h during gastric digestion. The maximum digestion of milk protein took place after intestinal phase. There was no significant difference between the digestive patterns of both variants ($P>0.05$). The highest degree of hydrolysis was observed in the intestinal extract of raw milk. The extent of proteolysis was least in intestinal extract of boiled milk followed by pasteurized milk. Whereas intestinal extract of sterilized milk showed more proteolysis compare to boiled milk. Peptide profile of water soluble extracts of intestinal extracts of milk processed at different temperature by RP-HPLC chromatogram showed that highest proteolysis was in raw milk followed by sterilized milk.

- To observe the effect of thermal processing on formation or degradation of BCM 7, three common treatments such as pasteurization, boiling and sterilization wereused for the processing of A1 and A2 cow milk.
- BCM 7 was not detected in A1 and A2 raw and processed milk from A1A1 and A2A2 genotype cows, respectively.
- As expected simulated *in- vitro* digestive study of raw milk and processed milk showed that 2 min oral digestion did not have any effect on milk proteins and no BCM 7 observed in salivary extract of all samples.
- Even gastric extracts of raw, pasteurized, boiled and sterilized milk did not showed BCM 7.
- Release of BCM 7 was observed in all samples of A1 and A2 raw cow milk after simulated *in-vitro* gastrointestinal digestion. Concentration of BCM 7 was 1399.32 ± 151.21 ng/ ml in A1 raw milk significantly higher compared to A2 milk (97.44 ± 21.04 ng/ ml) ($p<0.05$). Gastrointestinal extracts of all processed A1 cow milk samples exhibits about 14 -15 times higher content of BCM 7 compare to A2 milk.
- On comparing thermal processing treatments, gastrointestinal extracts of A1 raw milk showed highest release of BCM 7. Concentration of BCM 7 in gastrointestinal extract of milk within same genotype varies distinctly in the rangeof 74-146 ng/ ml in A2 and 900-1612 ng/ ml A1 milk.

- Presence of BCM 7 observed in gastrointestinal extracts of all pasteurized A1 and A2 milk. The content of BCM 7 in A2 cow milk was in the range of 46.0 -79.0 ng/ ml significantly lower than A1 milk (340-1132 ng/ml) ($P<0.05$). Concentration of BCM 7 in A2 and A1 pasteurized milk was lower than their counterpart in raw milk. About 50-52% reduction in release of BCM 7 was observed after LTLT pasteurization (63^0C for 30 min). An inhibitory effect of heat treatment on generation of BCM 7 was independent to the types of genotypes. About 73.00-77.00 % reduction in release of BCM 7 was observed in intestinal digestive extracts of boiled milk. The trend of reduced concentration was identical in both types of milk. Severity of thermal processing increased in sterilized milk so further reduction in release of BCM 7 was observed during intestinal digestion. The intestinal extracts of sterilized milk from both genotype cows showed the lowest concentration of BCM 7 compare to boiled and pasteurized milk. About 84.00 % reduction in the generation of BCM 7 observed during gastrointestinal digestion after giving sterilization treatment to both types of milk.
- Application of ELISA based assay for the quantification of BCM 7 in milk and milkproducts were used by a number of researcher, being a highly sensitive and easyto perform. Thereby to cross check the results of RP-HPLC methods,gastrointestinal digestive extracts of raw and processed milk were analyzed by ELISA assay. Identification and quantification of BCM 7 by ELISA assay also showed similar trend of occurrence of BCM 7 in all samples as identified by RP- HPLC method. Although concentrations of BCM 7 obtained by ELISA assay weresignificantly lower ($P<0.05$) than the concentration estimated by RP-HPLC method. As severity of heating enhanced concentrations of BCM 7 decreased in gastrointestinal extracts of processed milk i.e. sterilized milk showed lowestrelease of BCM 7 during simulated gastrointestinal digestion.
- MS analysis of lyophilized simulated crude *in–vitro* gastro-intestinal extract and specific HPLC fractions representative of BCM 7 of A1 and A2 raw milk was done using LCMS-MS. The results revealed that approximately 144 peptidessequences of β-casein were generated during

gastrointestinal digestion of raw milk and reported to cover 83% sequence coverage of β-CN molecule.

- Around 22 β-casein derived peptides were identified in the RP-HPLC fraction representing BCM 7 of intestinal extracts of A1 and A2 Milk. Number of peptides with varying length generated from β-CN fragment (especially from 54-72) during gastrointestinal digestion of milk from both genotype cows. Number of BCM 7 like peptides were released from β-CN molecule after gastrointestinal digestion of A2 milk, such as β (59-68), β (58-68), β (57-68), β (56-68), β (55-68) . These peptides contain more number of Pro residues. In addition to these peptides, we were able to identified number of BCM 7 like peptides in A2 milk after intestinal digestion i. e. BCM 7+1 (^{59}**VYPFPGPI**^{66}**)** and BCM 7+2 (^{58}**LVYPFPGPI**^{66}). Although thefrequency of occurrence of such peptides was less in A2.

- We did not able to identify BCM 7 in gastrointestinal extracts of A2 milk in spite ofpresence of number of pro BCMs and BCM 7 like peptide. Release of BCM 7 (^{60}**YPFPGPI**^{66}) and BCM 7 like peptides such as ^{59}**VYPFPGPI**^{66}, ^{53}**AQTQSLVYPFPGPI**^{66} and ^{54}**QTQSLVYPFPGPI**^{66} were identified during gastro- intestinal digestion of A1 milk and relative abundance of these types of peptides were higher in gastrointestinal extracts of A1 milk than A2 milk. However only two BCM 7 like peptides were identified (^{59}**VYPFPGPI**^{66} and ^{58}**LVYPFPGPI**^{66}) in A2 milk.

- The results obtained by RP-HPLC and ELISA showed the release of BCM 7 during gastrointestinal digestion of both types of milk. Whereas LCMS sequencing analysis provide the evidence that BCM 7 generated only in A1 milk during gastrointestinal digestion. Despite of occurrence of number of BCM 7 like peptides in gastrointestinal extracts, BCM 7 was not identified in A2 milk.

- To study the effect of fermentation process on formation and degradation of BCM7, *dahi* was prepared from A1 and A2 cow milk. Physicochemical characteristics of dahi prepared from A1 and A2 cow milk showed that there was no significant difference (P>0.05) between the proximate composition of *dahi* prepared from two different milk. pH, acidity, degree of proteolysis of both types of *dahi* were studied during storage at refrigerated temperature. As expected all samples of *dahi*showed a decline in pH values and increased in acidity with the storage period.

- In order to evaluate the relationship between extent of proteolysis and release of BCM 7, proteolysis in *dahi* samples during storage. The proteolysis was evaluated by measuring the changes in peptide content of water soluble extract and degree of hydrolysis of both types of dahi during storage period. The protein contents in the filtrate obtain by 12% TCA is related to the peptide concentration in WSE of the *dahi*. The peptide concentrations in both type of *dahi* were increased with storage time. No significant difference was observed between peptide content of A1 and A2 *dahi* (P>0.05). Extent of proteolysis in both types of *dahi* was determined by measuring the release of free amino acid residues in terms of L-leucine content. The rate of proteolysis or release of peptides increased with storage period in both types of *dahi.* Although there was no significant difference in the proteolytic activity between A1 and A2 *dahi* during storage at refrigeration condition (P>0.05).
- The PepX activities of prepared A1 and A2 *dahi* were evaluated during storage. There was an increase in PepX activity observed during storage. The increase in PepX activity was 8.09 - 13.36 nmol/ ml/ min in A2 *dahi* and 7.86 - 12.90 nmol/ ml/ min in A1 *dahi* respectively. Initially small increase of PepX activity observed up to 2 days thereafter rapid increased in activity observed with decrease in pH both types of *dahi*.
- *Dahi* prepared from A1 and A2 cow milk were analyzed for BCM 7 content by RP-HPLC method. BCM 7 was not identified in all samples of fresh and stored *dahi* up to one week.
- *Dahi* prepared from A1 and A2 cow milk subjected to simulated *in-vitro* digestion to study the effect of fermentation and gastrointestinal digestion on fate of BCM 7. Salivary extracts of all samples did not contain BCM 7. During gastric digestion release of BCM 7 was observed in both type of *dahi* prepared with A1 and A2 cow milk.
- MS analysis in earlier part of present study confirmed that along with BCM

7, a number of BCM 7 like peptides were released during simulated gastrointestinal digestion of A1 and A2 raw milk. It is better to refer as BCM 7 like peptides instead of BCM 7 in reporting the RP-HPLC results. Concentration of BCM 7 like peptides was very low and unable to quantify by RP-HPLC method in gastric extracts of both types of dahi. During storage it was observed that contents of BCM 7 like peptides were decreased in gastric extracts both type of dahi and disappeared in A2 *dahi* after 4 days of storage whereas in A1 *dahi* presence of these peptides observed until 6 days of storage.

- During simulated *in-vitro* gastrointestinal digestion of A1 and A2 *dahi*, BCM 7 like peptides were detected in all samples. The concentrations were significantlyhigher in A1 *dahi* than A2 *dahi* ($P<0.05$). About 4-5 times higher content of BCM 7 like peptides was observed in A1 *dahi* (281.35-896.51ng/ ml) than A2 *dahi* (82.35-191.40 ng/ ml).
- ELISA technique was used to cross validate the results obtained by RP-HPLC method for quantification of BCM 7 in *dahi* samples. BCM 7 like peptides were identified in gastric extracts of both types of *dahi* samples by ELISA assay. The concentration of BCM 7 in gastric extracts varied 10.00 - 18.0 ng/ml and 12.00-16.00 ng/ml in *dahi* prepared from A2 and A1 milk respectively. There was no significant difference in the release of BCM 7 with respect to the types of variants.
- Despite of presence of BCM 7 in all gastric samples we were unable to quantify with RP-HPLC method being low sensitivity of the method compare to ELISA. However RP-HPLC was able to provide the evidence of presence of BCM 7 likepeptides in *dahi* sample.
- Simulated *in-vitro* intestinal extracts of all samples were analyzed for BCM 7 by ELISA assay. Similar trend of release of BCM 7 after intestinal digestion was observed in both by RP-HPLC and ELISA. Concentrations of BCM 7 obtained by ELISA assay were significantly lower than the concentration evaluated by RP-HPLC method. Concentration of BCM 7 in fresh A2 *dahi* intestinal digestive extracts was 166.87 ng/ ml and 191.75 ng/ ml when calculated by ELISA and RP-HPLC method

respectively. In case of intestinal digestive extracts of A1 *dahi* BCM 7 concentration evaluated by ELISA and RP-HPLC was 590.26 and 896.00 ng/ml respectively.

- When stored samples were analyzed for BCM 7 after intestinal digestion by RP-HPLC and ELISA methods, it was observed that concentration of BCM 7 like peptides decreased with storage time significantly (P>0.05). There was nosignificant difference up to 2 days of storage. On 4th day of storage there was significant decrease of BCM 7 observed in all samples, whereas on 6th day of storage BCM 7 like peptides were identified only in the samples of A1 *dahi*. On 8th day pH of stored sample were very low near to 3.80 ± 0.12 in all samples with acidity 1.45 ± 0.10% and samples were showing syneresis and mould growth so further analysis for quantification of BCM 7 not carried out.
- To study the effect of ripening process Cheddar cheese was prepared from A1 and A2 cow milk. Proximate analysis revealed that moisture decreased as ripening proceeds. Initially first 4 months of ripening, decrease in moisture was not significant in both types of Cheddar cheeses prepared from A1 and A2 milk. After, 5 months changes in moisture content was significant in both types of cheese. Reduced moisture content in Cheddar cheese leads to increased levels of total protein and salt content. However fat content decreased as ripening progressed in both types of cheeses. As far as variant of milk concern used for the preparation of Cheddar cheese, all batches of cheese showed similar results and no significant difference was observed between proximate compositions of both types of cheeses ((p>0.05%).
- Cheddar cheese samples were analyzed for minerals such as sodium, calcium, potassium and phosphorous content using atomic absorption spectrophotometer. Content of all mineral remained nearly constant throughout ripening and no significant changes occurred during ripening period (p>0.05%). Among all estimated minerals, highest content of calcium was observed in both types Cheddar cheeses followed by sodium and phosphorous.

- Ripening had significant effect on acidity and pH of Cheddar cheese ($p<0.05\%$). Acidity increased with storage up to 5 months of storage period. After six months there was slight decrease in acidity observed in both types of Cheddar cheese. Gradual decrease in pH observed in both types of Cheddar cheese during ripening up to 5 months. After 5 months of ripening there was increase in pH value.
- To study the relationship between rate of proteolysis and formation of BCMs, extent of proteolysis was estimated by determining the ripening index to know the extent of primary and secondary proteolysis in Cheddar cheese prepared from A1 and A2 milk. The primary proteolysis was calculated by determining the ratio of nitrogen content of water soluble nitrogen to total nitrogen of cheese. Primary proteolysis indicates the release of large molecular weight peptides from caseins by the action of rennet (chymosin), milk enzymes and proteinases from starter culture. Ripening index (soluble nitrogen/total nitrogen (TN) x 100) increased 7.28 to 32.25. % and 8.74 to 31.13 % in A2 and A1 Cheddar cheese respectively during ripening period up to 9 months. Secondary proteolysis, expressed as the 12% TCA SN/TN, increased 2.19 to 16.12 and 3.40 to 16.57 % in A2 and A1 Cheddar cheese during ripening period up to 9 months. Excessive proteolysis observed in both type of Cheddar cheeses after four months of ripening, there was no significant difference observed between A2 and A1 cheese. 12% TCA SN/TN increased during ripening up to 9 months, but at a lower rate as compare to primary proteolysis. This fraction indicates the amount of small peptides and amino acids released by microbial proteinases and peptidases, mainly due to activity of starter and non-starter lactic acid bacteria and their enzymes.
- To study the effect of ripening on release of BCM 7 and BCM 7 like peptides in Cheddar cheese was evaluated up to 9 months of maturation at 3-4^{0}C. BCM 7 and BCM 7 like peptides were not released in fresh Cheddar cheese prepared from A1 and A2 milk. Even up to 3 months of storage release of BCMs did not observed in both types of cheeses. Presence of BCM 7 and BCM 7 like peptides was observed in A1 cheese

only after 4 months of ripening period. Up to 9 months these peptides were identified in A1 cheese and concentration of such peptides were in the range of 0.075-0.26 μg/g. Surprisingly presence of BCM 7 like peptides was also identified in A2 Cheddar cheese only after 7 months of ripening time. Concentration of such peptides in A2 cheese was in the range of 0.03-0.31 μg/g. There was no specific trend observed between ripening time and concentration of BCM 7 and BCM 7 like peptides in both types of Cheddar cheeses.

- After salivary phase similar results were obtained as reported above for undigested Cheddar cheese samples. As there was no proteolysis in oral phase so no effect on release of BCMs were seen in salivary phase of Cheddar cheese prepared from different type of milk.
- During gastric digestion there was no BCMs observed in fresh A1 and A2 Cheddar cheese but after 1 month of ripening BCM 7 like peptides were identified after gastric digestion of A1 Cheddar cheese. Whereas in gastric extracts of A2 Cheddar cheese presence of BCM 7 like peptides were seen after 2 months of ripening. Concentrations were higher in gastric extracts of A1 Cheddar cheese compare to A2 Cheddar cheese. There was no significant variation in BCM 7 likes peptides in gastric extracts of A2 Cheddar cheese throughout the ripening period and concentration varied between 0.58-1.88 μg/g. Contrary to these findings BCM 7 decreased during ripening in gastric extracts of A1 Cheddar cheese. Initially concentration of BCM 7 was 6.72 μg/g and it reduced to 3.09 μg/g after the nine months of ripening. About 50% reduction of BCM 7 like peptides observed during gastric digestion on completion of 9 months of ripening period.
- After simulated *in-vitro* gastrointestinal digestion, BCM 7 like peptides were observed in all intestinal extracts of A2 and A1 Cheddar cheese. The releases of BCM 7 like peptides were approximately 10 times higher in A1 than A2 Cheddar cheese. On comparison with undigested and gastric extracts it was observed that concentration of BCM 7 like peptides were higher in intestinal extracts irrespective to ripening time and variants

of β-CN present in Cheddar cheese.

- To evaluate the effect of ripening period on release or degradation of BCM 7 in Cheddar cheese, study was carried out up to 9 months. It was observed that concentration of BCM 7 decreased as ripening proceded especially in intestinal extracts. During initial stage of ripening concentration of BCM 7 like peptides were in range of 4.68 to 10.58 μg/g in A2 Cheddar cheese and 60.56-72.02 μg/g in A1 Cheddar cheese. Reduction in BCM 7 like peptides was not significant in A2 Cheddar cheese being low content of the peptides. As far as A1 Cheddar cheese concern changes in BCM 7 was significant with ripening period. In our study it was observed that reduction of BCM 7 like peptides were more significant after 3 months of ripening period, about 50 % reduction of these peptides took place. After 9 months of ripening concentration were 3.09 μg/g and 4.15 μg/g in A2 and A1 Cheddar cheese respectively. It was observed that after completion of ripening of 9 months concentration of BCM 7 like peptides in both Cheddar cheeses were nearly same. Significant reduction of BCM 7 like peptides were observed in A1 Cheddar cheese.
- ELISA technique was used to cross validate the results obtained by RP-HPLC method for quantification of BCM 7 in simulated gastrointestinal digestive extracts of A1 and A2 Cheddar cheese. Similar trend of release of BCM 7 was observed by ELISA assay also. Concentration of BCM 7 in gastrointestinal extracts of both types of Cheddar cheese obtained by ELISA assay weresignificantly lower than the concentration evaluated by RP-HPLC method in same products.
- To study the effect of ripening process on release of BCM 7 like peptides using ELISA assay, it was observed that release of BCM 7 peptide decreased after gastrointestinal digestion in both Cheddar cheese. Findings showed that concentration of BCM 7 like peptides decreased as ripening proceeds.
- To evaluate the effect of heat induced acid coagulation process, *Chhana* was prepared from A1 and A2. There was no significant difference

observed in proximate composition of both types of *Chhana* (p<0.05).

- Prepared *Chhana* was stored for one week at refrigerated condition (4-5^{0}C). Acidity increased with storage period in both types of *Chhana*, but no significant difference observed between A1 and A2 *Chhana* (p<0.05)
- To study the storage effect on stability of the product, FFA was estimated as index of rancidity. Even at low temperature storage FFA content increased in both types of samples but there was no significant difference observed betweenA1 and A2 *Chhana* (p<0.05) during storage .
- To evaluate the effect of coagulation on release of BCM 7, prepared *Chhana* was analyzed for the occurrence of BCM 7 before and after digestion using RP- HPLC method. BCM 7 and BCM 7 like peptides were not detected in *Chhana* prepared from A1 and A2 milk.
- There was no BCM 7 observed in the gastric extracts of *Chhana* prepared from A1 and A2 milk.
- Release of BCM 7 and BCM 7 like peptides were observed in all samples of *Chhana* prepared with A2 and A1 milk after simulated *in-vitro* gastrointestinal digestion. In freshly prepared *Chhana* content of BCM 7 and BCM 7 like peptides in A1 *Chhana* was 8.36 μg/ g about 2.5-3.0 times higher than A2 *Chhana* i.e. 3.34 μg/ g).
- *Chhana* samples were stored at refrigerated condition for one week and analyzed to study the release of BCM 7 and BCM 7 like peptides. During storage *Chhana* prepared from A1 milk showed higher concentration of BCM 7 like peptides than A2 *Chhana*. Content of BCM 7 like peptides decreased on storage up to 6 days of study. After 6 days of storage concentration of BCM 7 like peptides decreased to 0.48 and 1.56 μg/g in A2 and A1 *Chhana* respectively.
- BCM 7 in *Chhana* prepared from A2 and A1 milk were analyzed by ELISA assay. Identification and quantification of BCM 7 by ELISA assay also showed similar trend of occurrence of BCM 7 in all samples as identified by RP-HPLC method. Contradictory to other studies,

surprisingly there was no significant difference in the concentration of BCM 7 and BCM 7 like peptides evaluated by both methods.

- On comparision of release of BCM 7 in different milk products, It was observed that cocncentraion of BCM 7 was lowest in *dahi* samples and degradation of BCM 7 and alike peptides was higher than other dairy processing treatments as preparation of this products involves the combination of heating and fermentation. This finding indicates that fermentation process has greater effect on degradation of BCM 7. There by fermented dairy products can be considered as least hazardous products in terms of adverse effect of BCM 7.

Conclusion

From present study it can be inferred that BCM 7 does not release in A2 milk and milk products during gastrointestinal digestion, but presence of BCM 7 like peptides in the digestive extracts of A2 milk and milk products may contributes to the levels of BCM 7 during quantification by RP-HPLC and ELISA assay. Findings in this study indicate that release of BCM 7 and BCM 7 like peptides is more specific to milk from A1A1 genotype cow and required specific enzymes for their release. Although A2 variant of β-CN also able to generate BCM 7 like peptides but concentration is very low compare to their counterpart i.e. A1 variant. MS results support the hypothesis that BCM 7 peptides generates from the A1 β-CN variant. However BCM 7 like peptides can be generated during gastrointestinal digestion of A2 as well as A1 milk, but cleavage is more specific to His at '67' position compare to Pro at same position. Thermal processing such as pasteurization, boiling and sterilization has significant effect on the degradation of BCM 7 and BCM 7 like peptides. Other most popular process is fermentation also has a significant effect on the degradation of BCM 7 in fermented dairy products such as *dahi*. This process used LAB, has ability to further hydrolysis of BCM 7 and BCM 7 like peptides due to PepX activity. Cold storage condition and low pH favors cell lysis and releases more intracellular enzymes during storage of fermented products. There is least chance of release of BCM 7 in fermented products and further decreases with storage time. Hence fermented dairy products prepared from either milk may have little or no BCM 7/ BCM 7 like peptides. In addition to *Dahi* other most common fermented product is cheese involves long time ripening process. For the first time, present study was carried out in systematic way to evaluate the effect of ripening on release of BCM 7 like peptides in Cheddar cheeses and in their digestive extracts. It was observed that ripening has significant effect on release of BCM 7 like peptides, and so digestion process, especially amount of BCM 7 like peptides formed after intestinal digestion decreases as ripening proceed. This study indicates that after 9 months of ripening Cheddar cheese contain least concentration of BCM 7 like peptides. Heat induced acid coagulation also affect the release of BCM 7 especially after gastrointestinal digestion. Study carried out with *Chhana* showed that BCM 7 did not release during processing but this process helps in degradation of BCM 7 and BCM 7 like peptides with time.

Overall from present study we can draw an inference that different dairy processing conditions such as thermal, fermentation have significant effect on generation of BCM 7 and BCM 7 like peptides during gastrointestinal digestion of dairy products. Within the dairy processing condition fermentation shows highest degradation effects on BCM 7 and BCM 7 like peptides. Application of these techniques can reduced down the concentration of these peptides. Most of studies related to presence of BCM 7 were carried out in raw milk. Findings from our study indicate that dairy products prepared from milk containing either β-CN variant may be safe in term of adverse effect exerted by BCM 7 and BCM 7 like peptides especially fermented products. Present study was carried out with limited sample size, however results were reproducible. Further investigation is needed to confirm the effect of processing conditions on the formation and degradation of BCM 7 during digestion using high end techniques.

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