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Comparative evaluation of proteolytic activity of *Limosilactibacillus fermentum* and *Kluyveromyces marxianus* in whey protein supplemented media and its functional implications

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Abstract
This study evaluated the proteolytic activity of *Limosilactibacillus fermentum* and *Kluyveromyces marxianus* in whey protein-supplemented media. Proteolysis, a crucial fermentation process, breaks proteins into peptides with potential bioactivity. Both strains exhibited proteolytic activity, assessed via skim milk agar assay, microbial growth, protein content, and degree of hydrolysis. *Limosilactibacillus fermentum* demonstrated significantly higher proteolysis. The findings suggest that fermentation of whey protein by lactic acid bacteria and yeast could aid in the production of bioactive peptides with potential health benefits such as antioxidant, antihypertensive, or immunomodulatory effects, thereby promoting the use of whey in value-added functional food formulations.

Keywords: Roteolysis, *Limosilactibacillus fermentum*, *Kluyveromyces marxianus*, whey protein, bioactive peptides

Introduction
Proteolysis is the enzymatic cleavage of protein molecules into peptides and amino acids. It is a key process in both biological systems and food fermentation. In microbial fermentation, proteolytic activity refers to the ability of microorganisms to hydrolyze protein substrates, often contributing to improved flavor, texture, and bioactivity in fermented products (Tamang *et al.,* 2016) [18]. It not only influences the microbial nutrition and growth but also the generation of wide range of peptides with potential health benefits. Among various protein sources used in fermentation, whey protein is of particular interest due to its high biological value, abundance of essential amino acids—including sulfur-containing ones—and its crucial role in antioxidant activity (Rigamonti *et al.,* 2020) [15].
Fermentation of whey protein by lactic acid bacteria and yeast leads to the production of bioactive peptides with significant functional properties. In addition to its nutritional function, it also exhibits functional properties such as antihypertensive, antioxidant, and immunomodulatory effects (Fadimu *et al.*, 2022) [5]. Understanding the proteolytic efficiency of different microbial strains in whey protein supplemented media is vital to guide the development of functional foods targeting these health benefits.
Lactic acid bacteria (LAB) and yeast are widely used in food fermentation processes due to their capacity to modify sensory, nutritional, and preservative characteristics of food products. Their proteolytic systems vary significantly between species and strains, and are often influenced by the composition of the growth medium. LAB are widely recognized for their robust proteolytic systems, which support their growth in protein-rich environments and contribute to peptide production during fermentation (Leroy & De Vuyst, 2004) [9]. In yeasts such as *Kluyveromyces marxianus*, an extracellular proteolytic system that includes enzymes like proteinase A facilitates nitrogen assimilation and peptide generation during fermentation, thereby enhancing flavor, nutritional value, and interaction with co-cultured microorganisms (Fonseca *et al*., 2008; Reina-Posso & Gonzales-Zubiate, 2025) [6, 14].

The supplementation of culture media with whey protein presents an opportunity to stimulate microbial protease activity, which can facilitate protein hydrolysis and thus promote the generation of bioactive peptides with enhanced antioxidant, antimicrobial, ACE-inhibitory, and immunomodulatory properties (Zeng *et al.,* 2023) [20].

This study aims to evaluate and compare the proteolytic activity of selected yeast and LAB strains when cultured in whey protein-supplemented media. Studying the proteolytic activity of LAB and yeasts in whey protein-enriched media is helpful for understanding and comparing the effect and growth characteristics of these strains in protein rich media which is significant for both scientific understanding and industrial application. This approach not only promotes sustainable use of dairy waste but also supports the creation of functional foods with potential health benefits. The outcomes have direct relevance to the development of novel fermented foods, nutraceuticals, and sustainable bioprocesses.

Materials and Methods

Cultures used in the study

The LAB and the yeast cultures (*Lm.fermentum* and *Kl. marxianus*) were obtained from the culture stock of Department of Dairy Microbiology, College of Dairy Science and Technology, Pookode, Wayanad, Kerala, India. The activation of *Lm.fermentum* and *Kl.marxianus* were done by transferring to de Man Rogosa Sharpe (MRS) broth and Yeast Extract Peptone Dextrose broth respectively. The LAB were incubated at 37 ºC for 24h and yeast at 25 ºC for 48h. Three successive transfers of the cultures were done to ensure activity. The cultures were maintained and stored at -20 ºC in 20% glycerol. The purity and activity of the cultures were ensured by simple staining, Gram staining, catalase and oxidase tests (Barrow and Feltham,1993) [3].

Proteolytic activity

Skim milk agar was prepared by supplementing nutrient agar with skim milk (10% w/v) and was poured into sterile petri dish. Once the agar was solidified, a well (8 mm) was bored using a borer. To this 80 µl of the overnight grown culture was added and kept for incubation at 37 ℃ for 24 h for LAB and 25 ℃ for 24-72 h for yeast and observed for clear zones. Clear zones around the well indicated proteolytic activity of the culture and the diameter indicates the level of activity (Beganović *et al.*2013) [4].

Evaluation of growth in whey protein supplemented media

Preparation of whey based medium

Whey based media was prepared as per Aysha C.H *et al.*, 2012 [2] with slight modifications. Whey Protein concentrate (Fitness standard WPC-80, Mahaan milk foods Ltd, UP) was used in the study. The ingredients mentioned in the table was dissolved in freshly prepared whey and autoclaved.

Constituents	Quantity
Whey protein concentrate	15 g
Mineral mix:	
Dipotassium hydrogen phosphate	2 g
Magnesium sulphate heptahydrate	0.13 g
Manganese sulphate dihydrate	0.04 g
Tween 80	1ml
Whey	1000 ml

The culture was added at one per cent level and incubated at respective time temperature combinations and the sample were taken at 0, 24, 48 hours for the analysis.

Growth

The viable count of LAB and yeast in the samples were enumerated by pour plate method (ISO 4833-1:2014). Enumeration of LAB was done in MRS agar (37 ºC for 24 hr) and yeast in YPD agar (25 ºC for 48 hr). The average counts were taken and expressed in CFU/ml.

Titratable acidity

Titratable acidity of fermented whey protein supplemented media was assessed as per AOAC, 2019. Three to four drops of phenolphthalein indicator is added to 10 g of the sample. It is titrated against 0.1N NaOH solution till a pale pink colour appears. The titratable acidity was calculated as follows

Acidity as % of lactic acid = $(Titre\ value \times Normality\ of\ NaOH \times 90 \times 100) \div (weight\ of\ samp(g) \times 1000)$

Degree of hydrolysis by OPA (o-phthaldialdehyde) method

Degree of hydrolysis of whey protein supplemented media was done by OPA method (Nielsen *et al*, 2001) [11]. Three ml of OPA reagent was added to test tubes. 400 µl of the sample were added and kept exactly for two min before measuring. For blank, 400 µl of deionised water was added. For standard 400 µl serine standard was used. Double determination of each sample was done along with four tubes for standard and four tubes for blank. The absorbance at 340 nm was measured using a UV-Vis spectrophotometer (Agilent, USA). Deionised water was used as the control and the assay was done at room temperature. The two standards were measured before sample, followed by two blanks and also at the end, after measuring all samples, the remaining standard and blanks were measured and their mean was taken. The typical OD of standard was 0.8 and blank 0.7.

Calculation

Serine-NH_2= $OD_{sample} - OD_{blank}$ / $OD_{standard}$ -OD_{blank} *0.9516 meqv/L * 0.1 * 100/X*P

X= g sample
P= protein %
0.1 is the sample volume in litre (L)
h= (serine-NH_2-β)/α meqv/g protein
α=1.00 for whey
β=0.40 for whey
DH = h/h_{tot} * 100%
h_{tot}=8.8 for whey

Determination of protein content by Lowry method

The estimation of protein content was done using Lowry method (Lowry *et al.*, 1951, Olson *et al.*, 2007) [10, 17]. One ml of the sample was taken in a test tube and five ml of freshly prepared Lowry assay mix was added. After keeping the tubes at room temperature for ten minutes add 0.5 ml of diluted Folin-Ciocalteu reagent and vortex immediately. Then the tubes were kept for 30 min at room temperature. The absorbance of the samples were measured at 660nm.

Statistical analysis
The data obtained in the experiment will be analysed statistically (Snedecor and Cochran, 1994) [17] using the SPSS software version 22.

Results
The *Lm.fermentum* cultures were gram positive, catalase negative and oxidase negative. The yeast *Kl.marxianus* showed oval to ellipsoidal shape on simple staining.

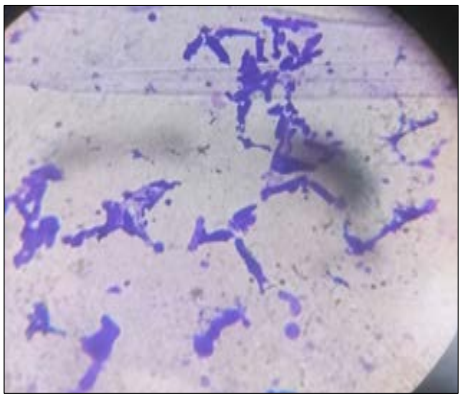


Fig 1: Kl. Marxianus

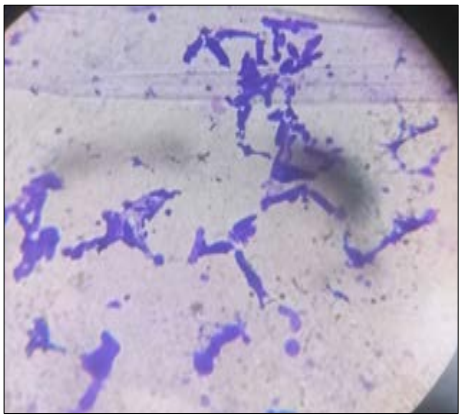
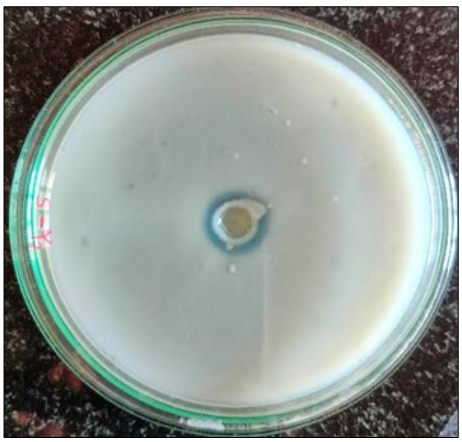


Fig 2: Lm. fermentum

Proteolytic activity
Presence of clear zone around the inoculated well indicated proteolysis. The quantification was done by measuring the diameter of zone of clearance. Both the samples showed proteolytic activity. The zone formed by *Lm. fermentum* was 20.5 ±.70 mm and of *Kl. marxianus* is 5 ± 0.70mm.



Kl. marxianus

Lm. Fermentum

Growth
The growth of the organisms were assessed by enumerating the viable count in log CFU/ml by pour plating method. The growth measured of *Lm.fermentum* and *Kl.marxianus* is shown in fig.3.

Strain	0th hr	24th hr	48th hr
Lm. fermentum	8.494 ± 0.592	11.733 ± 1.030	12.157 ± 0.686
Kl. marxianus	7.065 ± 0.091	11.286 ± 1.335	10.231± 0.8605

Titratable acidity
The titratable acidity(%LA) of the samples at specified intervals were as follows

Organism	0th hr	24th hr	48th hr
Lm. fermentum	0.399 ± 0.10	0.759 ± 0.041	0.951 ± 0.010
Kl. marxianus	0.387 ± 0.0	0.639 ± 0.0	0.855± 0.0

Fig 4 represents the titratable acidity of *Lm.fermentum* and *Kl.marxianus.* At 0th hour, both the medium shows similar titratable acidity. As the incubation proceeds, the acidity increases for both *Lm.fermentum* and *Kl.marxianus* indicating the growth and utilisation of media to produce metabolites.

Degree of hydrolysis by OPA
The degree of hydrolysis (%) was measured by OPA method at the intervals of 0,24 and 48 hours of incubation. Fig 5 represents the degree of hydrolysis of *Lm.fermentum* and *Kl.marxianus.* It also indicates the increment of degree of hydrolysis over incubation and *Lm.fermentum* giving the maximum percent of degree of hydrolysis.

Organism	0th hr	24th hr	48th hr
Lm. fermentum	12.31	18.46	35.89
Kl. marxianus	10.51	25.21	29.56

Estimation of protein by Lowry method
Protein content in the sample was estimated by Lowry method. The incubated samples were analysed at 0, 24 and 48 hours of interval. Fig 6 represents the estimated protein of *Lm.fermentum* and *Kl.marxianus.* The protein content (mg/ml) observed are as follows,

Organism	0th hr	24th hr	48th hr
Lm. fermentum	52.83	53.29	58.45
Kl. marxianus	48.61	47.24	46.73

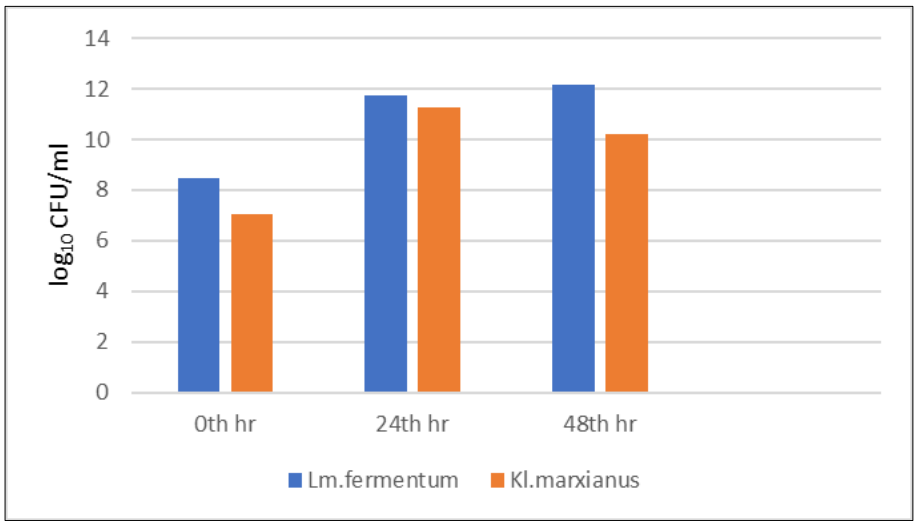


Fig 3: Growth

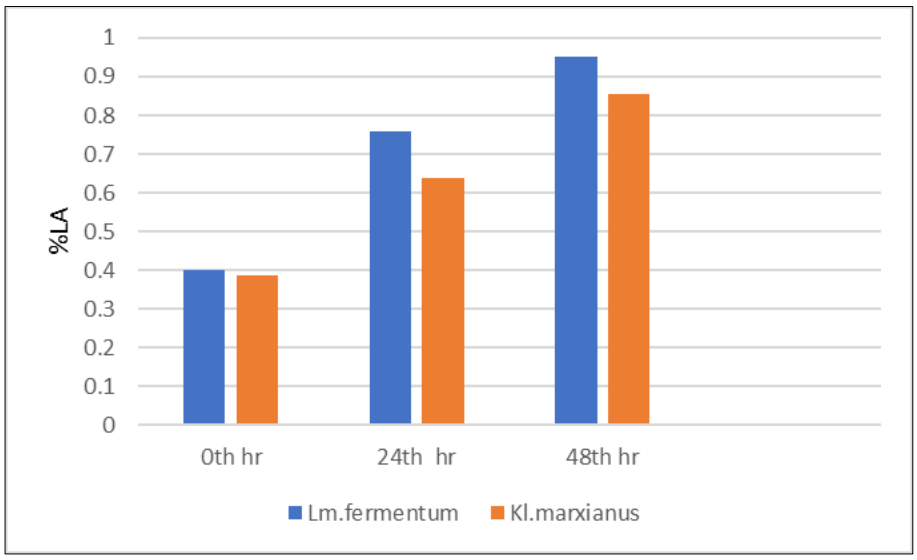


Fig 4: Titratable acidity

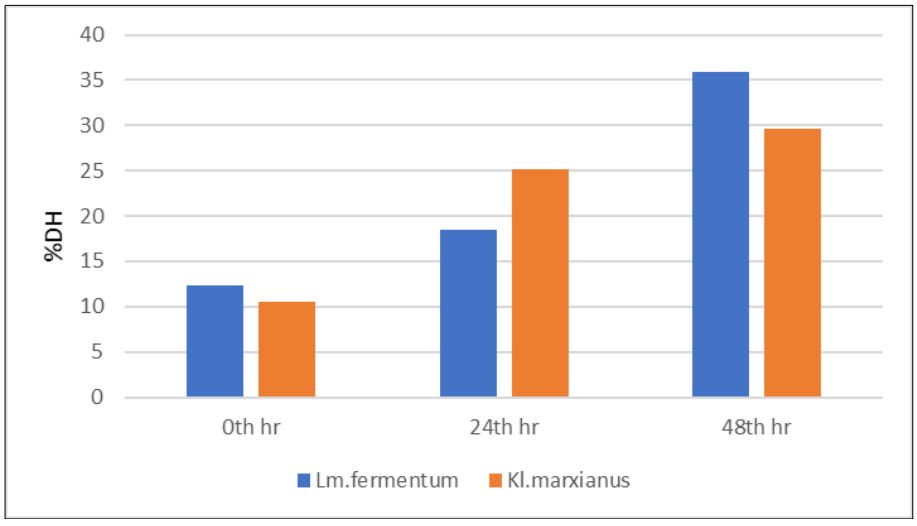


Fig 5: Degree of Hydrolysis

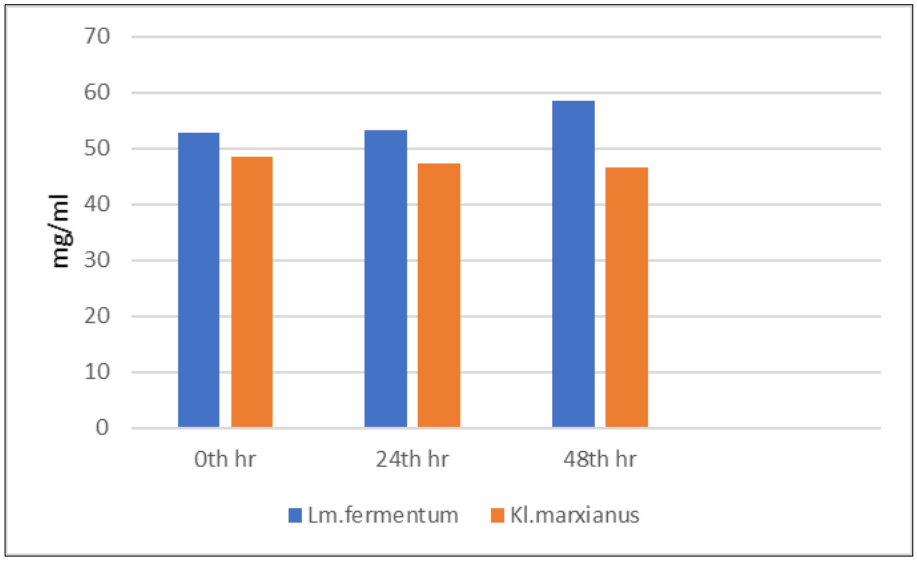


Fig 6: Estimation of protein

Discussion

The present study investigated and compared the proteolytic activity of *Limosilactibacillus fermentum* and *Kluyveromyces marxianus* in whey protein-supplemented media. Evaluation parameters included microbial growth, titratable acidity, degree of protein hydrolysis, and residual protein content over 0, 24, and 48 hours of fermentation.

Both strains demonstrated the ability to grow in whey protein-based medium, with *Lm. fermentum* exhibiting a consistent increase in viable count throughout the incubation period. In contrast, *Kl. marxianus* showed an initial rise in viable count up to 24 hours, followed by a modest decline at 48 hours. This decline may be attributed to nutrient depletion, acid accumulation, or metabolic stress during extended fermentation, which is commonly observed in yeast fermentations under static conditions (Leroy & De Vuyst, 2004) [9].

The titratable acidity of the fermented samples increased progressively for both strains, indicating active metabolic conversion of substrates and organic acid production. *Lm. fermentum*, showed higher final acidity, which aligns with its lactic acid-producing capabilities. Acid production during fermentation not only influences microbial growth and viability but also affects the solubility and hydrolysis pattern of whey proteins, potentially enhancing peptide release.

Proteolytic activity, assessed through zone of clearance in skim milk agar, was significantly higher for *Lm. fermentum* (20.5 ± 0.70 mm) compared to *K. marxianus* (5.0 ± 0.70 mm). This observation is consistent with previous reports that LAB possess well-developed proteolytic systems, including cell-envelope-associated proteinases and intracellular peptidases, which effectively degrade milk proteins into peptides and amino acids (Savijoki *et al*., 2006) [16]. Although yeast demonstrated relatively lower proteolytic activity, its ability to hydrolyze whey proteins is notable. *K. marxianus* possesses extracellular proteases such as proteinase A, which contribute to nitrogen assimilation and release of peptides during fermentation (Van Den Berg *et al*., 1996; Fonseca *et al*., 2008) [19, 6].

The degree of hydrolysis, measured using the OPA method, showed a steady increase for both strains, with *Lm. fermentum* reaching 35.89% and *K. marxianus* 29.56% at 48 hours. This confirms the proteolytic breakdown of whey proteins over time and supports the formation of low molecular weight peptides. Notably, the yeast exhibited a rapid increase in hydrolysis at 24 hours, indicating early-phase enzymatic activity, followed by a plateau, possibly due to feedback inhibition or protease denaturation.

Protein content analysis revealed an increase in *Lm. fermentum*-fermented samples, which may suggest accumulation of peptide-rich hydrolysates, while a slight decline was observed in *K. marxianus*-treated samples. The reduction in protein concentration in yeast-fermented media could reflect further breakdown of peptides into free amino acids or assimilation by yeast cells.

Overall, the findings highlight the superior proteolytic potential of *Lm. fermentum* compared to *K. marxianus* in whey protein-supplemented systems. The increased degree of hydrolysis and peptide formation by LAB is of particular interest in the context of developing functional dairy-based foods. Previous research has demonstrated that peptides generated during whey fermentation may exhibit a range of bioactivities including ACE-inhibition, antioxidant, antimicrobial, immunomodulatory, and anti-inflammatory effects (Korhonen & Pihlanto, 2006; Olvera-Rosales *et al*., 2022) [8, 13].

The current study underscores the importance of microbial selection for targeted proteolysis and bioactive peptide production. While LAB show promising potential for generating functional hydrolysates from whey, the role of yeast such as *K. marxianus* in contributing flavor, aroma, and auxiliary bioactivity merits further investigation, particularly in co-culture systems. Future research should focus on peptide characterization through peptidomics and functional validation using *in vitro* or *in vivo* models to fully explore the health-promoting potential of fermented whey protein products.

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