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Research paper

Comparative effects of bacterial proteases on the physicochemical stability of cream liqueur

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ABSTRACT

Heat-stable microbial proteases secreted by psychrotrophs in raw milk can survive pasteurisation and compromise dairy emulsion stability, yet their behaviour in complex matrices such as cream liqueur is poorly understood. This study compared three bacterial proteases (AprX from *Pseudomonas fluorescens* and HhoA from two *Bacillus cereus* strains) and evaluated their effects on a commercial Irish cream liqueur during 20 d of ambient storage, alongside reconstituted skim milk. Stability was assessed by viscosity, colour, particle size distribution, and casein hydrolysis. Protease activity was matrix-dependent: HhoA was most active in skim milk (rapid gelation at 37°C), whereas AprX predominated in cream liqueur. By Day 20, AprX caused 67.1% casein hydrolysis, increased Dx90 from 0.193 to 0.340 μm, broadened the size distribution (span >3.0), and shifted colour ($\Delta E = 12.8$). A biphasic viscosity response suggested significant hydrolysis of caseins followed by aggregation. Overall, ethanol–fat–caseinate interactions modulated protease behaviour and identified AprX-producing *Pseudomonas* as a key enzymatic risk to cream liqueur stability.

1. Introduction

Cream liqueurs are commercially significant oil-in-water emulsions comprising 10–16% butterfat, 15–20% (v/v) ethanol, and 2.5–4.0% sodium caseinate as primary emulsifiers (Banks & Muir, 1985; Euston & Goff, 2019; Heffernan et al., 2009; Lynch & Mulvihill, 1997). Product stability depends critically upon sodium caseinate integrity at the oil-water interface, providing steric and electrostatic stabilisation (Dickinson et al., 1989).

Cream liqueurs are formulated to be intrinsically resistant to microbial spoilage: the combined hurdles of 15–20% (v/v) ethanol, reduced water activity from added sugar, and trisodium citrate addition together produce an environment in which neither *Pseudomonas* spp. Nor *Bacillus* spp. Can grow, supporting the typical two-year ambient shelf life of commercial products such as Irish cream liqueurs (Banks et al., 1981; Lynch & Mulvihill, 1997). The principal proteolytic risk to product stability therefore does not arise from microbial growth in the bottled product, but from heat-stable extracellular proteases that are secreted in the raw milk or cream during refrigerated storage prior to processing and that subsequently survive pasteurisation, carrying over into the finished liqueur as a residual enzymatic activity.

Psychrotrophic *Pseudomonas* spp. Routinely become the dominant flora of cold-stored raw milk and secrete AprX at cell densities of $\geq 10^6$ CFU mL^{-1} (Machado et al., 2017; Marchand et al., 2009), while *Bacillus* spore-formers contribute additional caseinolytic activity, including HhoA (De Jonghe et al., 2010; Yang et al., 2021). Both enzyme classes show exceptional thermostability; AprX in particular has been shown to retain activity following UHT treatment (140°C, 4 s) and to undergo partial reactivation during subsequent storage (Dufour et al., 2008; Stoeckel et al., 2016), meaning that the heat treatment applied to the dairy fraction during cream liqueur manufacture is unlikely to fully inactivate any enzyme already present. Once incorporated into the bottled product, these enzymes can act, slowly but progressively, with the potential to compromise interfacial stabilisation, initiate age gelation analogous to that observed in UHT milk (Anema, 2019; Datta & Deeth, 2001), and produce the viscosity drift, sediment, and colour change occasionally reported as long-term stability failures of cream-based liqueurs.

The metalloprotease AprX from *Pseudomonas fluorescens* exhibits exceptional thermostability (Dufour et al., 2008; Marchand et al., 2009; Stoeckel et al., 2016), and exhibits chymosin-like activity, preferentially cleaving κ-casein at (or very close to) the chymosin-sensitive region around Phe_{105}–Met_{106}, thereby releasing CMP-like fragments (including "pseudo-CMP") and destabilising casein micelles (Matéos et al., 2015;

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Baglinière et al., 2013; D'Incecco et al., 2019). This initiates age gelation through progressive casein hydrolysis and peptide aggregation (Anema, 2019; Datta & Deeth, 2001; Rauh et al., 2014; Zhang et al., 2018; D'Incecco et al., 2019). Although AprX is most frequently characterised in terms of its chymosin-like cleavage of κ-casein at the Phe_{105}–Met_{106} bond (Matéos et al., 2015; Baglinière et al., 2013; D'Incecco et al., 2019), the enzyme exhibits broad-specificity caseinolytic activity and has been shown to hydrolyse α_{s1}-, α_{s2}-, and β-casein in addition to κ-casein, particularly during prolonged storage (Mu et al., 2009; Zhang et al., 2018). The relative contribution of κ-casein versus α_s- and β-casein hydrolysis to overall destabilisation may itself be matrix-dependent, with the unfolded, non-micellar caseinate organisation in cream liqueur potentially exposing α_s- and β-casein to enzymatic hydrolysis to a greater extent than in native micellar systems. Similarly, *Bacillus cereus* HhoA protease demonstrates significant thermostability and caseinolytic activity (Contesini et al., 2018; De Jonghe et al., 2010; Gopal et al., 2015; Meng et al., 2022; Porcellato et al., 2021; Yang et al., 2021).

Despite extensive research in conventional dairy systems, protease behaviour in cream liqueur remains unexplored. The cream liqueur matrix differs fundamentally from milk: ethanol alters protein conformation and solubility (Feng et al., 2021; Mezdour et al., 2008; Nikolaidis et al., 2017); fat may enhance *Pseudomonas* protease activity (Zhang et al., 2020); and sodium caseinate exists as dissociated molecules rather than native micelles (Broyard & Gaucheron, 2015). These factors may differentially modulate enzyme activity in ways unpredictable from simpler systems.

In this study, AprX and HhoA were produced in reconstituted skim milk, and the resulting cell-free, heat-stable enzyme preparations were then introduced into a commercial Irish cream liqueur to simulate residual proteolytic activity entering the bottled product after pasteurisation. The proteolytic activities of AprX (*P. fluorescens*) and HhoA (*B. cereus*) were compared in reconstituted skim milk and in cream liqueur, and their effects on physicochemical stability were evaluated through viscosity, particle size distribution, colour, and SDS-PAGE measurements. To our knowledge, this represents the first systematic comparison of bacterial protease behaviour between conventional milk and cream liqueur systems, and the first quantitative assessment of the proteolytic risk that pre-secreted psychrotrophic proteases pose to cream liqueur stability.

2. Materials and methods

2.1. Materials

Skim milk powder (SMP; protein content 34.5%, lactose 52.1%, fat <1.5%) was obtained from Ornua Ingredients (Dublin, Ireland). Commercial Irish cream liqueur was sourced from an Irish manufacturer. The composition, as declared by the manufacturer, was: 15.7% fat (w/w), 17.0% ethanol (v/v), 38.2% total solids (w/w), and protein (primarily as sodium caseinate) ≈3.0% (w/w). The remaining ≈19.5% (w/w) of total solids comprised added sucrose (≈14% w/w), residual lactose carried over from the dairy fraction (≈3% w/w), trisodium citrate added as a calcium-chelating stabiliser (≈0.15% w/w), and minor non-fat dairy solids, flavouring, and caramel colour. Of these, sucrose and residual lactose are reducing-sugar substrates relevant to Maillard-type browning during ambient storage, and trisodium citrate is mechanistically relevant to the activity of the calcium-dependent metalloprotease AprX (see Section 4). Specific values for the proprietary fractions were not disclosed by the manufacturer; the values stated above are typical for Irish cream liqueurs of this composition class (Banks et al., 1981; Heffernan et al., 2009; Lynch & Mulvihill, 1997).

Azocasein was obtained from Megazymes (Bray, Ireland). Sodium azide, aprotinin (from bovine lung, 3–7 TIU/mg protein), trichloroacetic acid (TCA), Tris-HCl, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Laemmli sample buffer, Coomassie Brilliant Blue R-250, and molecular weight markers (Precision Plus Protein™ Dual Colour Standards, 10–250 kDa) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Bio-Rad precast Mini-PROTEAN® TGX™ gels (12% acrylamide) were from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals used were of analytical reagent grade.

2.2. Bacterial strains and protease preparation

Pseudomonas fluorescens strain NCIMB 9046, originally isolated from pre-incubated raw milk, was obtained from the National Collection of Industrial, Marine and Food Bacteria (Aberdeen, UK). *Bacillus cereus* strains NCTC 11143 (designated B1) and NCIMB 9373 (designated B2) were obtained from the National Collection of Type Cultures (Public Health England, London, UK) and NCIMB, respectively. All strains were maintained on nutrient agar at 4°C with monthly subculturing, and working stocks were stored at −80°C in 20% (v/v) glycerol.

2.2.1. AprX extraction

AprX-containing supernatant was prepared following the method of Paludetti et al. (2020) with modifications. *P. fluorescens* NCIMB 9046 was cultured on nutrient agar at 25°C for 48 h. Colonies were transferred to 10% (w/w) reconstituted SMP (100 mL in 250 mL Erlenmeyer flasks) and incubated at 28°C with orbital shaking (150 rpm) for 7 days to allow maximum protease secretion. The culture was centrifuged at 20,000 × *g* for 30 min at 20°C (Sorvall RC 6 Plus; Thermo Fisher Scientific, Waltham, MA, USA), and the cell-free supernatant containing extracellular AprX was collected, filter-sterilised (0.22 μm; Millipore, Burlington, MA, USA), and stored at −20°C until use. The preparation was designated "AprX extract" and used within 4 weeks of preparation.

Enzymes were intentionally produced in reconstituted skim milk rather than cream liqueur because *Pseudomonas* and *Bacillus* psychrotrophs do not grow in the alcoholic, low-water-activity environment of the finished liqueur; skim milk represents the physiologically relevant upstream matrix in which these organisms produce AprX and HhoA during cold storage of raw milk and cream, and the cell-free, heat-stable supernatants therefore approximate the residual enzymatic load that survives pasteurisation of the dairy fraction and is carried into the bottled product.

2.2.2. HhoA extraction from Bacillus cereus

HhoA-containing supernatants were prepared from *B. cereus* strains NCTC 11143 and NCIMB 9373. Cultures were inoculated from overnight nutrient broth cultures into 10% reconstituted SMP and incubated at 30°C without shaking until visible decrease in opacity indicated substantial proteolysis (typically 48–72 h). Cell-free supernatants were obtained by centrifugation at 20,000 × *g* for 30 min, filter-sterilised, and stored at −20°C. A third strain (NCDO 577) was initially screened but excluded from later trials due to weak proteolytic activity in preliminary assessments.

2.3. Enzymatic activity standardisation

Proteolytic activities were quantified and standardised using the azocasein assay (Christen & Marshall, 1984) with modifications optimised for comparative analysis. Azocasein solution (1% w/v) was prepared by dissolving azocasein in sterile phosphate buffer (20 mM K_2HPO_4/KH_2PO_4, pH 7.45) with gentle warming (50°C, 30 min) and overnight stirring at 4°C to ensure complete dissolution. The solution was filter-sterilised and stored at 4°C protected from light.

Enzyme extract (50 μL) was combined with azocasein solution (250 μL) in 1.5 mL microcentrifuge tubes and incubated at 37°C for 60 min in a thermomixer (Eppendorf, Hamburg, Germany) without shaking. Reactions were terminated by addition of 300 μL TCA solution (5% w/v), which precipitates undigested azocasein and high-molecular-weight peptides. After standing at room temperature for 10 min protected from light, samples were centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant (300 μL) was carefully transferred to fresh tubes, combined

with equal volume of Tris-HCl buffer (0.1 M, pH 7.5) and absorbance was measured at 440 nm (Cary 60 UV-Vis spectrophotometer; Agilent Technologies, Santa Clara, CA, USA). Blank controls received TCA prior to enzyme addition. All measurements were performed in triplicate.

Proteolytic activity was expressed as ΔA_{440} (absorbance increase relative to blank). Relative activities were calculated using *B. cereus* NCTC 11143 as reference (exhibiting highest activity in preliminary skim milk trials). Working doses were determined by inverse proportionality to relative activity, anchored on BC11143 at 0.40 μL mL^{-1}:

$$Dose_x = Dose_{BC11143} / Relative\ Activity_x$$

Final working doses for skim milk trials were: AprX extract = 1.63 μL mL^{-1}; *B. cereus NCTC* 11143 = 0.40 μL mL^{-1}; *B. cereus NCIMB* 9373 = 0.68 μL mL^{-1}. For cream liqueur trials, doses were doubled to account for anticipated matrix effects (see Section 2.5).

This standardisation step was performed to ensure that, after dilution, each enzyme extract delivered an equivalent effective proteolytic activity in the reaction system. Because the crude extracts differed in initial activity, working doses were adjusted by inverse proportionality to the azocasein-derived relative activity so that comparisons reflected enzyme behaviour in the matrix rather than differences in starting activity.

2.4. Skim milk trial design

Reconstituted skim milk (10% w/w) was prepared by dissolving SMP in deionised water with continuous magnetic stirring (500 rpm, 30 min, 20°C) until complete dissolution was visually confirmed. Sodium azide (0.1% w/v final concentration) was added to prevent microbial growth, and aprotinin (0.01 mg mL^{-1}) was included to inhibit indigenous plasmin activity. The reconstituted milk was equilibrated at 20°C for 2 h before enzyme addition.

Samples (10 mL) were allocated to five treatment groups in sterile polypropylene tubes (15 mL; Sarstedt, Nümbrecht, Germany): Control (no enzyme addition), AprX, B1 (*B. cereus* NCTC 11143), and B2 (*B. cereus* NCIMB 9373). Three replicate samples from each treatment were incubated at each of three temperatures: 4°C (refrigeration), 20°C (ambient), and 37°C (accelerated). At Days 1, 4, and 7, samples were visually assessed for destabilisation and 2 mL aliquots removed and stored at −20°C for subsequent SDS-PAGE analysis. The complete trial was performed in duplicate (n = 2 independent trials).

2.5. Cream liqueur trial design

Based on preliminary trials indicating reduced enzyme efficacy in the cream liqueur matrix (no rapid destabilisation observed at skim milk dosages), enzyme dosages were doubled to achieve measurable destabilisation within a practical experimental timeframe. Final dosages were: AprX extract = 3.26 μL mL^{-1}; B1 = 0.80 μL mL^{-1}; B2 = 1.36 μL mL^{-1}.

Commercial cream liqueur was gently mixed by repeated inversion (not vortexing, to avoid foaming) and dispensed into sterile 120 mL polypropylene screw-cap containers (100 mL per container). All handling was performed at ambient temperature under aseptic conditions. Enzyme extracts were added, containers were sealed, gently inverted three times to ensure uniform distribution, and stored at ambient temperature (20 ± 2°C) in darkness to prevent light-induced oxidation.

Sampling was performed at Days 0, 5, 10, 15, and 20. At each time point, containers were gently inverted three times before removing aliquots for analysis of viscosity (5 mL), colour (10 mL), particle size (approximately 0.5 mL), and SDS-PAGE (2 mL). SDS-PAGE samples were immediately frozen at −20°C; other analyses were performed within 2 h of sampling. Three independent trials were conducted (n = 3), with triplicate measurements within each trial.

2.6. Analytical methods

2.6.1. Visual assessment

Samples were visually examined at each time point for evidence of destabilisation, including changes in opacity, colour, viscosity (qualitative flow assessment), phase separation, precipitation, and gelation. Observations were recorded using standardised terminology: “normal” (no visible change), “slight precipitation” (fine particles visible), “precipitation” (distinct sediment), “soft gelation” (increased viscosity, sample flows slowly when inverted), “compact gelation” (sample holds shape briefly when inverted), and “coagulation” (firm gel, no flow when inverted).

2.6.2. Instrumental colour measurement

Colour was measured using a Minolta CR-400 Chroma Meter (Konica Minolta, Tokyo, Japan) equipped with a CR-A33a glass light projection tube and calibrated against a standard white tile (Y = 94.0, x = 0.3130, y = 0.3191) before each measurement session. Samples (10 mL) were transferred to optical glass cuvettes and measurements performed at 20°C. CIE L* (lightness, 0 = black, 100 = white), a* (−60 = green, +60 = red), and b* (−60 = blue, +60 = yellow) coordinates were recorded (D65 illuminant, 2° standard observer). Each sample was measured in triplicate. Total colour difference (ΔE) relative to Day 0 was calculated as: $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5}$. Values of $\Delta E > 1.0$ are considered perceptible by trained observers, while $\Delta E > 3.0$ indicates commercially significant colour change.

2.6.3. Viscosity measurement

Apparent viscosity was determined using an Anton Paar ViscoQC™ 100 rotational viscometer (Anton Paar, Graz, Austria) fitted with an appropriate small-volume measuring system and spindle. Samples (≈6.7 mL) were equilibrated at 20 ± 0.5°C in the measurement chamber connected to a circulating water bath for 5 min prior to analysis. Viscosity was measured at a fixed rotational speed of 100 rpm and readings were recorded after 30 s of continuous rotation to ensure steady-state conditions. Three aliquots were analysed for each sample.

2.6.4. Particle size distribution

Particle size distribution was determined by laser diffraction using a Malvern Mastersizer 3000 (Malvern Panalytical Ltd., Malvern, UK) equipped with a Hydro MV wet dispersion unit. The optical model employed a refractive index of 1.46 for milk fat globules (imaginary component 0.001) and 1.33 for the dispersant (deionised water at 20°C). Samples were added dropwise to the dispersion unit (stirring speed 1250 rpm, no ultrasonication to preserve aggregate structure) until laser obscuration reached 6–8%. Each sample was then measured three times consecutively without re-dispersion, with each measurement comprising 10 s red laser and 10 s blue laser acquisition. Particle size distributions were calculated from the average scattering signal of the three measurements.

Parameters reported include: $D_x(10)$, $D_x(50)$, $D_x(90)$ (diameters below which 10%, 50%, and 90% of the particle volume exists, respectively); D [4,3] (volume-weighted mean diameter, De Brouckere mean); D [3,2] (surface-weighted mean diameter, Sauter mean); and Span $[(D_x(90) - D_x(10))/D_x(50)]$, a dimensionless measure of distribution breadth. Measurements were performed in triplicate.

2.6.5. SDS-PAGE analysis

Protein hydrolysis was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions following a modified Laemmli protocol (Blakesley and Boezi, 1977). It is acknowledged that, under non-reducing conditions, intact κ-casein migrates as a heterogeneous distribution of disulphide-linked oligomers rather than as a discrete monomeric band, and the para-κ-casein cleavage product is not cleanly resolved; consequently, percentage breakdown values reported in this study refer specifically to the

combined α_s-casein and β-casein bands, and direct quantification of κ-casein hydrolysis was not performed.

I. Sample preparation for skim milk: Frozen samples were thawed at room temperature. Aliquots (100 μL) were diluted 1:5 (v/v) with deionised water in microcentrifuge tubes. Diluted sample (100 μL) was combined with 2× Laemmli sample buffer (100 μL; containing 4% SDS, 20% glycerol, 0.004% bromophenol blue, 125 mM Tris-HCl pH 6.8) and heated at 90°C for 3 min with intermittent vortexing. Samples were cooled to room temperature before loading.
II. Sample preparation for cream liqueur: Fat was removed prior to electrophoretic analysis using a freeze-thaw extraction protocol. Samples (2 mL) were centrifuged at 14,000 rpm (Eppendorf 5424 R) for 30 min at 40°C to liquefy fat and enhance density-driven separation. Tubes were immediately transferred to −20°C for 15 min to solidify the fat layer, which was then carefully removed by aspiration with a spatula. This cycle was repeated 1–3 times until negligible fat remained. The defatted aqueous phase was collected using a syringe from below any residual fat layer, diluted 1:5 with deionised water, and processed as described for skim milk samples.
III. Electrophoresis: Samples (10 μL) were loaded onto precast 12% Mini-PROTEAN® TGX™ gels alongside Precision Plus Protein™ molecular weight markers. Electrophoresis was performed in Tris-glycine-SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at constant voltage (100 V) for approximately 130 min until the dye front reached the gel base. Gels were fixed in 40% methanol/10% acetic acid for 30 min, stained with Coomassie Brilliant Blue R-250 (0.1% in 40% methanol/10% acetic acid) for 1 h, and destained in 40% methanol/10% acetic acid until the background was clear.
IV. Densitometry: Stained gels were imaged using GelAnalyzer software version 23.1.1 (http://www.gelanalyzer.com/) under white epi-illumination. Band intensities were quantified using ImageJ software (version 1.53; NIH, Bethesda, MD, USA). For each lane, regions of interest were manually defined around α_s-casein and β-casein bands, and integrated density was measured after local background subtraction. Total casein was calculated as the sum of α_s-casein and β-casein band intensities. Percentage breakdown was calculated relative to Day 0 control samples within each gel: $\text{Breakdown (\%)} = [(\text{Intensity}_{\text{Day 0}} - \text{Intensity}_{\text{Day n}})/\text{Intensity}_{\text{Day 0}}] \times 100$.

2.7. Statistical analysis

All quantitative data are expressed as mean ± standard deviation of measurements from three independent trials (n = 3), with triplicate measurements within each trial. Statistical analyses were performed using IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA). Normality was assessed using the Shapiro-Wilk test, and homogeneity of variance using Levene's test. For normally distributed data with homogeneous variance, one-way analysis of variance (ANOVA) was applied to assess effects of enzyme treatment within each time point, and effects of storage time within each treatment. *Post-hoc* pairwise comparisons were conducted using Tukey's honestly significant difference (HSD) test. For data violating ANOVA assumptions, the Kruskal-Wallis test with Dunn's *post-hoc* comparison was applied. Two-way ANOVA was used to examine interactions between enzyme treatment and storage time. Statistical significance was set at $\alpha = 0.05$. Different lowercase superscript letters indicate significant differences ($p < 0.05$) among treatments at the same time point.

3. Results

3.1. Skim milk trial: baseline characterisation of proteolytic activity

3.1.1. Visual assessment of destabilisation

Visual assessment of reconstituted skim milk samples revealed clear temperature-dependent and enzyme-specific patterns of destabilisation (Table 1). At 4°C, all treatments remained visually stable throughout the 7-day storage period.

At 20°C, differential enzyme effects became apparent by Day 4. *B. cereus* proteases (B1 and B2) induced soft gelation, characterised by notably increased viscosity and slow flow upon inversion, while AprX-treated samples remained fluid. By Day 7 at 20°C, AprX-treated samples exhibited compact gelation, and both *B. cereus* treatments progressed to coagulation with firm gel formation.

The most pronounced effects occurred at 37°C, where *B. cereus* proteases rapidly induced soft gelation within 24 h, progressing to complete coagulation by Day 4. AprX-treated samples followed a similar trajectory with slightly delayed onset, reaching coagulation by Day 7.

3.1.2. SDS-PAGE analysis of skim milk

SDS-PAGE analysis provided quantitative confirmation of visual observations (Fig. 1). At 37°C after 7 days (representing maximum proteolysis), AprX treatment resulted in 94.6% breakdown of α_s-casein bands relative to Day 0 control, with substantial hydrolysis of β-casein also evident. Both *B. cereus* proteases caused extensive proteolysis, with B2 causing the most pronounced breakdown, i.e. 62.1% reduction of β-casein and 27.0% reduction of α_s-casein as quantified by densitometry. The appearance of lower molecular weight bands (15–20 kDa region) indicated accumulation of casein-derived peptides.

3.2. Cream liqueur trial: physical stability assessment

3.2.1. Visual observations

The cream liqueur trial revealed markedly different patterns of enzyme activity compared with skim milk, with the hierarchy of protease efficacy substantially altered by the complex matrix; however, in contrast to the overt destabilisation observed visually in skim milk, the visual differences between treatments in the cream liqueur trial were considerably more subtle, with the most pronounced changes emerging only when samples were characterised instrumentally (Sections 3.3–3.6).

Visual assessment after 20 days of ambient storage (Fig. 2) showed

Table 1
Instability map of reconstituted skim milk (10% w/w) with added bacterial proteases during 7 days of storage at 4°C, 20°C, and 37°C (Trial 1). Cell shading indicates severity of destabilisation. Protease concentrations were standardised using the azocasein assay. AprX was extracted from *Pseudomonas fluorescens* NCIMB 9046; *B. cereus* proteases (HhoA) were extracted from strains NCTC 11143 and NCIMB 9373.

Storage Time	Temp.	Control	AprX (1.63 μL/mL)	*B. cereus* NCTC 11143 (0.4 μL/mL)	*B. cereus* NCIMB 9373 (0.68 μL/mL)
Day 1	**4°C**	✓	✓	✓	✓
	20°C	✓	✓	✓	+
	37°C	✓	+	✓	++
Day 4	**4°C**	✓	✓	✓	✓
	20°C	✓	✓	✓	×
	37°C	✓	++	×	×
Day 7	**4°C**	✓	✓	✓	✓
	20°C	✓	××	××	××
	37°C	✓	⨯	⨯	⨯

Legend: Destabilisation Status

✓	Stable	+	Slight precipitation	++	Precipitation
×	Soft gelation	××	Compact gelation	⨯	Coagulation

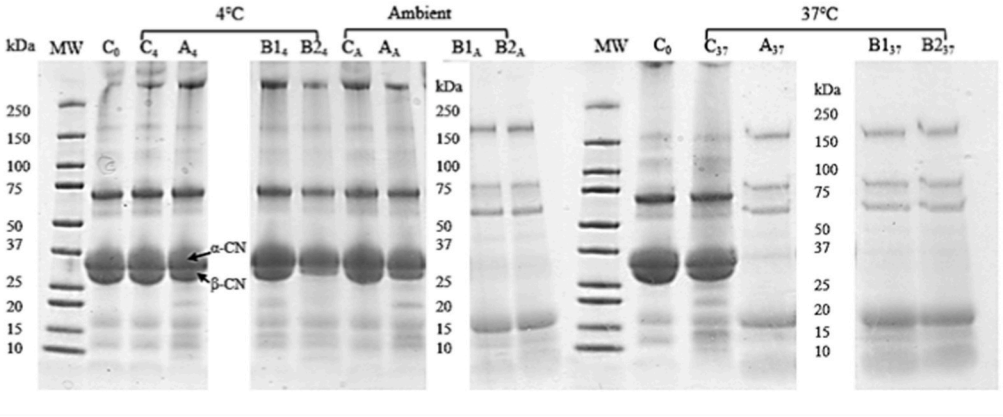


Fig. 1. SDS-PAGE gel electrophoretogram of skim milk powder (SMP) samples incubated for 7 days under different conditions. Lanes labeled MW represent the Molecular Weight Standard. Lane C_0 corresponds to SMP control at Day 0. Lanes C represent SMP control stored at 4°C, ambient temperature, or 37°C. Lanes A correspond to SMP with AprX enzyme, lanes B1 to SMP inoculated with HhoA NCTC 11143, and lanes B2 to SMP inoculated with HhoA NCIMB 9373, under the same storage conditions.

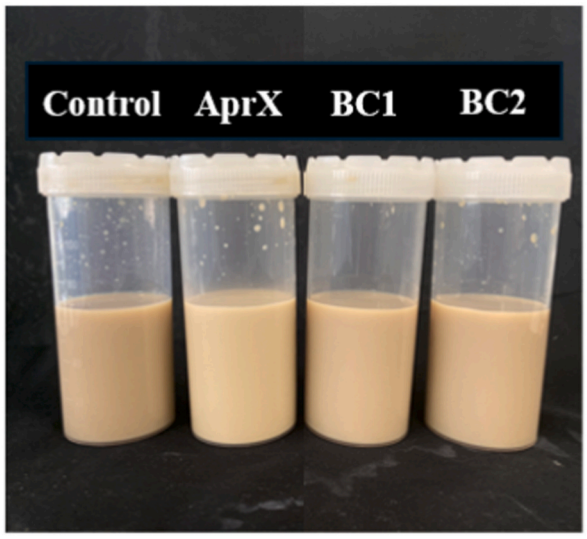


Fig. 2. Visual appearance of cream liqueur samples after 20 days of ambient storage (20 ± 2°C). From left to right: Control (no enzyme), AprX, *B. cereus* NCTC 11143 (BC1), and *B. cereus* NCIMB 9373 (BC2). Note pronounced discolouration, phase separation, and textural changes in AprX-treated sample (second from left). Representative images from Trial 2.

that the AprX-treated sample exhibited a shift in colour from the darker cream-white appearance of the control to a paler, yellow-brown tone. The samples treated with *B. cereus* (B1 and B2) showed only marginal visual differences relative to the control, with no clear colour change apparent. Control samples retained the typical cream-white appearance and showed no visually evident change in colour or texture over the 20-day storage period. A slightly clarified serum layer and minor textural irregularity on gentle inversion were noted in the AprX treated samples during bulk handling.

3.3. Colour changes in cream liqueur

Instrumental colour analysis quantified the visually apparent changes (Fig. 3). At Day 0, all samples exhibited comparable colour profiles characteristic of fresh cream liqueur: L* ≈58 (moderate lightness), a* ≈ 0.8 (essentially neutral on the green-red axis), and b* ≈ 13.7 (moderate yellowness attributable to the fat phase and any carotenoid pigments).

AprX-treated samples showed progressive and substantial colour changes throughout storage. The L* value increased from 58.2 ± 0.05 at Day 0 to 69.0 ± 0.11 at Day 20 and 69.4 ± 0.03 at Day 30, representing an increase in lightness. The a × value increased from 0.78 ± 0.02 to 1.75 ± 0.02 (Day 20) and 2.03 ± 0.03 (Day 30), indicating a shift toward red. The b × value rose from 13.7 ± 0.03 to 17.6 ± 0.12 (Day 20) and 19.7 ± 0.03 (Day 30), indicating enhanced yellowness. The total colour difference (ΔE) reached 11.6 ± 0.3 at Day 20 and 12.8 ± 0.2 at Day 30, values substantially exceeding the threshold for commercial acceptability (typically $\Delta E < 3.0$ for dairy products).

In contrast, the *B. cereus* protease–treated samples (B1 and B2) showed only minor colour changes that closely tracked the Control treatment throughout the 20-day storage period. By Day 20, B1-treated samples showed small increases in L* (58.2 → 59.1), a* (0.78 → 0.91), and b* (13.7 → 14.2), corresponding to a total colour difference of $\Delta E = 1.10 \pm 0.08$ relative to Day 0. B2-treated samples showed slightly smaller shifts (L* 58.2 → 58.9, a × 0.78 → 0.88, b × 13.7 → 14.1; $\Delta E = 0.88 \pm 0.06$). Control samples showed minimal change over the same period (L* 58.2 → 58.6, a × 0.78 → 0.83, b × 13.7 → 13.9; $\Delta E = 0.51 \pm 0.04$). All three of these treatments (Control, B1, B2) remained well below the $\Delta E > 3.0$ commercial-significance threshold and were not statistically distinguishable from one another at Day 20 ($p > 0.05$), although B1 was marginally elevated relative to Control.

3.4. Viscosity changes in cream liqueur

Viscosity measurements revealed characteristic biphasic behaviour in AprX-treated samples that was highly reproducible across three independent trials (Fig. 4). At Day 0, all treatments exhibited statistically comparable apparent viscosities ranging from 18.18 ± 0.08 to 18.34 ± 0.15 mPa s ($p > 0.05$), confirming uniform initial conditions and indicating that enzyme addition did not cause immediate changes in bulk rheological properties.

By Day 10, a pronounced and statistically significant ($p < 0.001$) decrease in viscosity was observed in AprX-treated samples, declining to 15.49 ± 0.18 mPa·s-representing a 15.8% reduction from initial values. The *B. cereus* protease treatments exhibited moderate viscosity decreases at Day 10: B1 declined to 16.78 ± 0.09 mPa s (8.5% reduction, $p < 0.01$) and B2 to 17.41 ± 0.19 mPa s (4.4% reduction, $p < 0.05$). Control (18.38 ± 0.17 mPa s) samples showed no significant change from Day 0.

By Day 20, AprX-treated samples showed a strong increase in viscosity (19.90 ± 1.99 mPa s), approximately 8.5% higher than at the start, with large variation between repeats. B1 samples showed continued viscosity reduction at Day 20 (16.14 ± 0.08 mPa s),

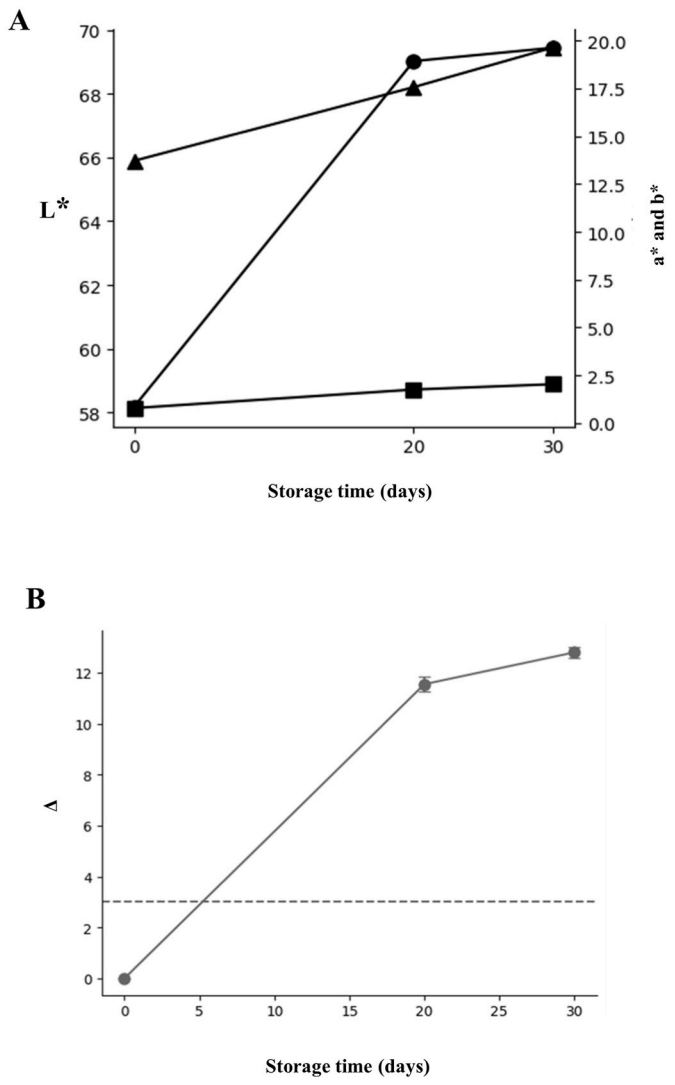


Fig. 3. Changes in CIE colour parameters of AprX-treated cream liqueur during 30 days of ambient storage. (A) L* (●), a* (■), and b* (▲) values as a function of storage time. (B) Total colour difference (ΔE) relative to Day 0. Symbols represent mean values and vertical bars indicate ± standard deviation (n = 9); due to the small magnitude of variability relative to the scale of the axes, error bars are visually subtle. The dashed horizontal line in (B) denotes the threshold for commercially perceptible colour change (ΔE = 3.0). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

remaining significantly lower than control ($p < 0.001$).

3.5. Particle size distribution in cream liqueur

Laser diffraction analysis of particle size distribution provided quantitative evidence of emulsion destabilisation that complemented and corroborated viscosity measurements (Fig. 5). At Day 0, all cream liqueur samples exhibited narrow, monomodal particle size distributions characteristic of a well-stabilised emulsion.

By Day 10, AprX-treated samples showed early signs of destabilisation, with statistically significant ($p < 0.05$) increases in $D_x(90)$ to 0.206 ± 0.001 μm (6.7% increase) and Span to 1.401 ± 0.050 (8.2% increase), indicating the onset of aggregation and/or coalescence. Other treatments maintained Day 0 values: Control (Span = 1.298 ± 0.003), B1 (1.298 ± 0.003), and B2 (1.296 ± 0.007), none significantly different from Day 0 ($p > 0.05$).

After 20 days, AprX-treated samples exhibited dramatic

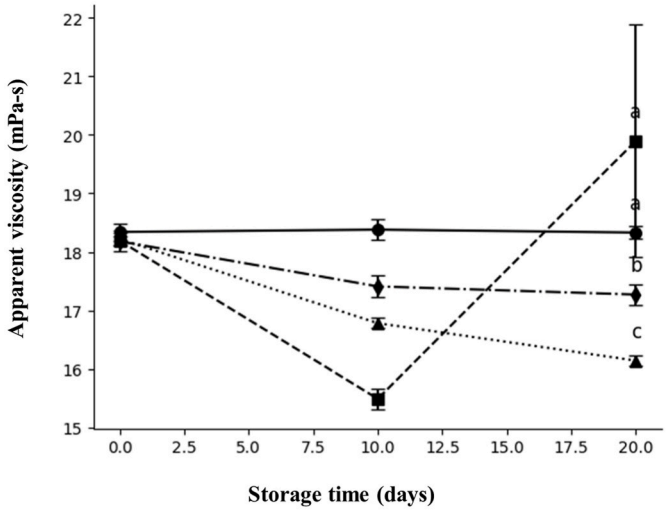


Fig. 4. Changes in apparent viscosity (mPa·s) of cream liqueur samples during 20 days of ambient storage. (A) Viscosity profiles for all treatments: control (●, solid line), AprX (■, dashed line), BC11143 (B1; ▲, dotted line), and BC9373 (B2; ◆, dash–dot line). Values are means ± SD. Lowercase letters indicate significant differences among treatments at Day 20 ($p < 0.05$, Tukey's HSD).

destabilisation with profound changes in all particle size parameters. $D_x(90)$ increased to 0.340 ± 0.010 μm (76% increase from Day 0). Concurrently, $D_x(10)$ decreased from 0.053 to 0.026 ± 0.000 μm (51% decrease). D [3,2] (surface-weighted mean) decreased from 0.091 to 0.062 ± 0.001 μm, reflecting the increased contribution of small particles to surface area. Span increased to 3.193 ± 0.015-a 147% increase indicative of severe polydispersity and heterogeneous destabilisation.

B. cereus protease treatments showed minimal particle size changes over 20 days, with B1 showing slight broadening (Span = 1.330 ± 0.030 at Day 20) but B2 remaining essentially unchanged (Span = 1.303 ± 0.002).

3.6. SDS-PAGE analysis of casein hydrolysis in cream liqueur

SDS-PAGE analysis provided direct molecular-level evidence of proteolytic activity and enabled quantification of casein breakdown over the 20-day storage period (Fig. 6; Table 2). At Day 5, control samples exhibited limited hydrolysis (7.7% reduction in total casein band intensity), likely representing proteolysis due to indigenous enzymes, e.g., plasmin. AprX-treated samples already showed substantial proteolysis, with 40.0% reduction in combined α_s-casein and β-casein band intensities relative to Day 0 control. B1 (19.2%) and B2 (18.1%) showed moderate but comparable levels of early hydrolysis (see Fig. 7).

Samples treated with AprX maintained the highest activity throughout storage: 60.8% breakdown at Day 10, 61.4% at Day 15, and 67.1 ± 2.3% at Day 20. Pronounced breakdown of α_s-casein (≈30–32 kDa region) and β-casein (≈25 kDa region) was accompanied by appearance of low-molecular-weight bands (10–15 kDa) representing accumulated hydrolysis products.*B. cereus* proteases showed more modest progression. B1 reached 30.2% breakdown at Day 10 and 36.1% at Day 20, while B2 plateaued at approximately 22–24% from Day 10 onwards.

3.7. Correlation of analytical parameters

For AprX-treated samples, Pearson correlation analysis revealed significant positive correlations between casein breakdown (%) and Span ($r = 0.94, p < 0.01$), ΔE ($r = 0.97, p < 0.01$) and $D_x(90)$ ($r = 0.91, p < 0.01$; Table 2). The relationship between casein breakdown and viscosity showed a negative correlation during the thinning phase (Days 0–10; r = −0.89) and positive correlation during the thickening phase

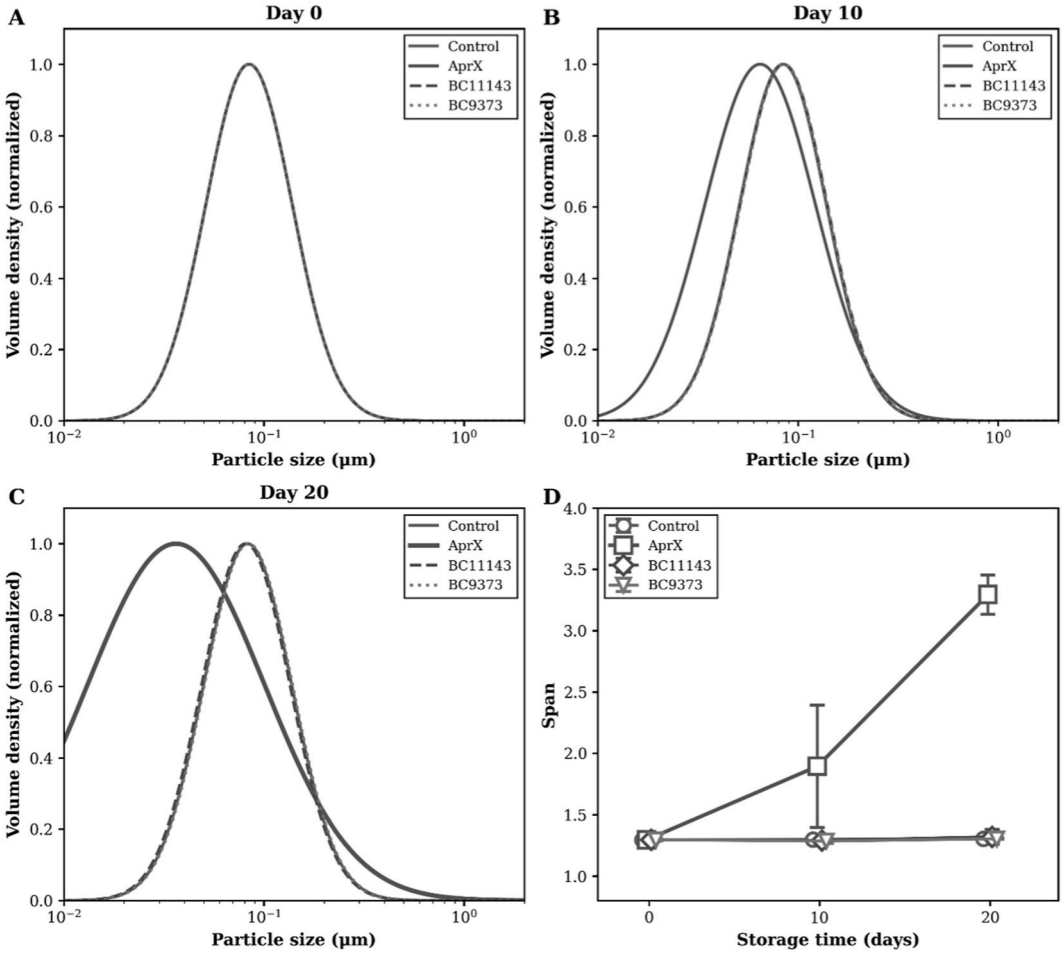


Fig. 5. Particle size distributions of cream liqueur samples during ambient storage. (A) Distribution curves at Day 0 (all samples overlaid). (B) Distribution curves at Day 10. (C) Distribution curves at Day 20; note the pronounced broadening and development of bimodal character in AprX-treated samples. (D) Changes in Span parameter [(Dx (90) − Dx (10))/Dx (50)] over time for all treatments. Distribution curves represent averages of triplicate measurements. Error bars in Panel D represent standard deviation (n = 9).

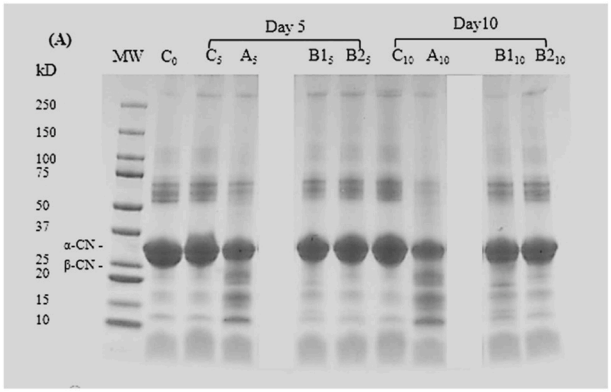
(Days 10–20; r = +0.76).

4. Discussion

In skim milk, the stability observed across all treatments at 4°C is consistent with the expected suppression of enzymatic activity at refrigeration temperatures, arising from reduced molecular mobility and enzyme kinetics that follow Arrhenius behaviour (Q_{10} typically 1.5–2.5 for proteases) (Rao et al., 1998). At higher tempeartures, under azocasein-standardised conditions, *B. cereus* HhoA proteases exhibited the highest destabilising activity, followed by AprX, a hierarchy consistent with previous reports identifying HhoA as a highly active caseinolytic enzyme in milk systems (Meng et al., 2022; Yang et al., 2021).

The reversal of this hierarchy upon transition to cream liqueur-from *B. cereus* > AprX in skim milk to AprX ≫ *B. cereus* in cream liqueur-represents a central finding of this study with important implications for quality control strategies. The multiple physical and chemical parameters measured in the cream liqueur trial collectively support a coherent, mechanistically linked model of AprX-driven destabilisation: the simultaneous increase in L*, a*, and b × colour coordinates can be explained by enhanced light scattering from aggregated and coalesced fat globules and from casein-derived peptide aggregates, observations analogous to those of Zhang et al. (2018) in AprX-treated UHT milk, while the increases in redness and yellowness further reflect fat phase exposure as emulsion structure breaks down, potential Maillard reaction between liberated casein-derived amino acids (particularly lysine ε-amino groups) and reducing sugars yielding brown melanoidins, and altered light-scattering characteristics changing the relative contributions of reflection and absorption.

The biphasic viscosity response-initial thinning followed by recovery-provides rheological evidence consistent with the κ-casein-cleavage destabilisation mechanism characterised for AprX in UHT milk (Matéos et al., 2015; Baglinière et al., 2013; D'Incecco et al., 2019; Anema, 2019). Although κ-casein was not directly quantified in this study, the thinning phase might be attributed to the cleavage at the Phe_{105}–Met_{106} bond and release of caseinomacropeptide (CMP, ~6.8 kDa) or pseudo-glycomacropeptide into the serum phase (D'Incecco et al., 2019), whereupon the hydrophilic CMP no longer contributes to interfacial stabilisation or bulk viscosity and the remaining *para*-κ-casein segment loses its protective function, allowing destabilised casein and fat particles to undergo initial aggregation without immediately forming a continuous network, the subsequent viscosity recovery at Day 20, accompanied by high inter-replicate variability, is linked to secondary aggregation driven by hydrophobic forces and calcium bridges, forming a weak gel-like structure (Zhang et al., 2018), with the variability likely reflecting the stochastic nature of gel formation near the gelation threshold. This biphasic behaviour is fully consistent with the age gelation cascade established in UHT milk (Anema, 2019; Datta & Deeth, 2001), demonstrating that an analogous process operates in cream liqueur despite its substantially different matrix composition and confirming AprX as a potent destabilising agent in alcoholic dairy



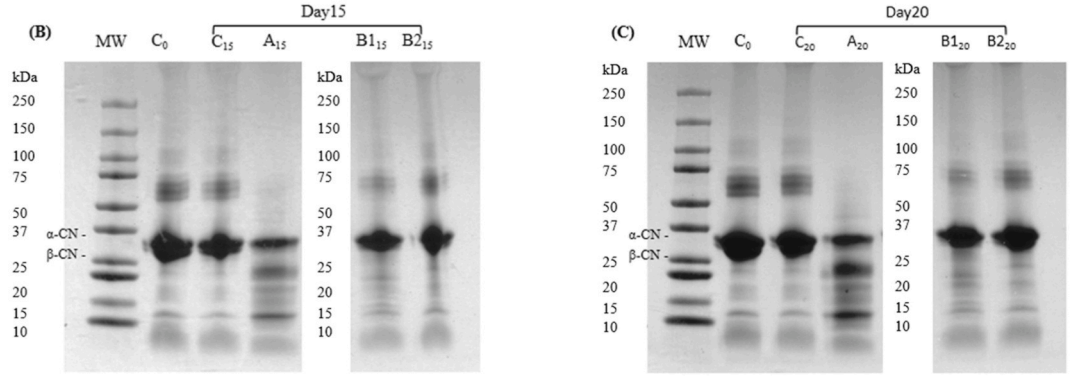


Fig. 6. SDS-PAGE gel electrophoretograms of cream liqueur (CL) samples incubated at time points (A) Day 5 and Day 10, (B) Day 15, and (C) Day 20 (Representative of Trial 2). Lanes labeled MW represent the Molecular Weight Standard. Lane C_0 corresponds to CL control at Day 0. Lanes C represent CL control at different incubation times. Lanes A correspond to CL with AprX enzyme, lanes B1 to CL inoculated with HhoA NCTC 11143, and lanes B2 to CL inoculated with HhoA NCIMB 9373. α-Casein and β-casein bands are indicated on the gel.

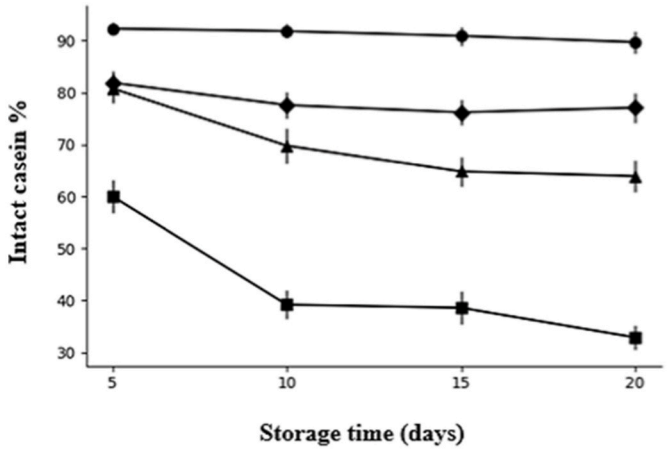


Fig. 7. Intact casein remaining (%) (α_s-casein + β-casein) in cream liqueur during ambient storage, quantified by SDS-PAGE densitometry. Values are expressed as the percentage remaining relative to Day 0. Data are shown for Trial 2 for the control (●), AprX (■), BC11143 (B1; ▲), and BC9373 (B2; ◆) treatments. Symbols represent mean values and vertical bars indicate ± standard deviation (n = 3).

emulsions.

The particle size data corroborate this mechanistic picture: AprX-mediated proteolysis of caseins might cause internal structure breakdown and removal of caseinomacropeptide from the κ-casein monolayer at the fat globule surface (Dickinson et al., 1989) which eliminates steric stabilisation, promoting flocculation and coalescence (Baglinière et al., 2013; Zhang et al., 2020), as evidenced by the 76% increase in $D_x(90)$ by Day 20, while the concurrent 51% decrease in $D_x(10)$ and reduction in D [3,2] indicate simultaneous formation of small casein-derived peptide aggregates in the serum phase, together producing the 147% increase in Span that reflects severe polydispersity and heterogeneous destabilisation; correspondingly, Pearson correlations for AprX-treated samples confirmed that casein breakdown, particle size broadening, colour change, and rheological alteration are mechanistically linked

Table 2
Pearson correlation coefficients between analytical parameters in AprX-treated cream liqueur samples during 20 days of ambient storage. Correlations were calculated from measurements at Days 0, 5, 10, 15, and 20. Viscosity correlation with casein breakdown showed biphasic behaviour: negative correlation during initial thinning (Days 0-10) and positive correlation during recovery phase (Days 10-20).

Parameter pair	r	p-value	Interpretation
Casein breakdown vs Span	0.94	<0.01	Strong positive
Casein breakdown vs ΔE	0.97	<0.01	Strong positive
Casein breakdown vs Dx (90)	0.91	<0.01	Strong positive
Casein breakdown vs Viscosity (Days 0-10)	−0.89	<0.05	Strong negative (thinning phase)
Casein breakdown vs Viscosity (Days 10-20)	+0.76	<0.05	Moderate positive (recovery phase)
Span vs ΔE	0.92	<0.01	Strong positive
Span vs Dx (90)	0.96	<0.01	Strong positive
Delta E vs Dx (90)	0.89	<0.01	Strong positive

probably through the common pathway of cleavage of caseins ($r = 0.94$ for Span, 0.97 for ΔE, and 0.91 for $D_x(90)$, all $p < 0.01$), and this correlated multiparameter response suggests that a single non-destructive measure such as colour could serve as a practical predictive indicator of overall stability in quality control applications (Table 2). The SDS-PAGE data further confirm this picture, showing that AprX had substantially depleted accessible substrate by Day 10 (60.8% breakdown), with only modest further hydrolysis through Day 20 (67.1 ± 2.3%), suggesting that later-stage physical destabilisation is driven not by continuing enzymatic activity but by the aggregation and gelation cascade already initiated.

While κ-casein cleavage almost certainly contributes to the destabilisation, the present data demonstrate that α_s- and β-casein hydrolysis represents a quantitatively major component of the proteolytic destabilisation in this matrix. In cream liqueur, sodium caseinate exists as dissociated, unfolded molecules adsorbed at the oil-water interface or as small aggregates in the continuous phase (Broyard & Gaucheron, 2015), rather than as native micelles where α_s- and β-casein are largely buried in the hydrophobic core and shielded from solvent-exposed proteolysis. The non-micellar caseinate organisation in cream liqueur may therefore expose α_s- and β-casein to AprX to a greater degree than in conventional milk systems, broadening the substrate specificity of the enzyme as it actually operates in this product matrix.

In contrast, activity of *B. cereus* proteases plateaued at substantially lower hydrolysis levels (B1: 36.1%; B2: ~22–24% by Day 20), and their persistent viscosity thinning without recovery suggests progressive emulsion breakdown that does not generate conditions favouring secondary aggregation within the experimental timeframe-possibly reflecting different peptide profiles produced by HhoA compared to AprX, or the lower overall extent of hydrolysis. Several non-mutually exclusive mechanisms may account for this attenuated *Bacillus* protease activity in cream liqueur: ethanol at 17% (v/v) may cause partial denaturation or conformational change in HhoA by disrupting the hydrophobic interactions that stabilise its tertiary structure and by altering active site geometry (Feng et al., 2021), whereas AprX, as a zinc-dependent metalloprotease with a calcium-stabilised serralysin-family structure, may be inherently more resistant to alcohol-induced unfolding. The structural organisation of casein also differs fundamentally between the two matrices-HhoA may have optimal activity against native micellar casein in skim milk, while in cream liqueur, caseinate exists as unfolded molecules adsorbed at fat globule surfaces or as small aggregates in the continuous phase, a presentation potentially suboptimal for HhoA binding and catalysis; the high fat content (15.7%) creates extensive oil-water interfacial area that may favour AprX activity through interfacial concentration or conformational activation, as demonstrated by Zhang et al. (2020), while physically sequestering casein in a way that limits accessibility to *Bacillus* proteases that may not efficiently partition to the interface; the substantially reduced free calcium in cream liqueur-arising from the use of sodium caseinate and intentional citrate addition for emulsion stability (Power, 1996)-would be expected to impair AprX activity given its calcium-dependent catalytic mechanism, yet sufficient calcium may be locally available from progressive casein hydrolysis or via the dairy cream fraction, whereas differential calcium chelation and ionic strength effects may disproportionately impair HhoA stability or substrate binding; finally, the high sugar content (12–16%) reduces free water availability and increases viscosity, slowing enzyme diffusion and substrate access, with enzymes requiring greater conformational mobility or substrate searching likely most affected.

Together, these matrix effects explain why AprX retains its destabilising potency through a combination of structural resistance to alcohol-induced denaturation, preferential action at the κ-casein Phe_{105}–Met_{106} bond at interfacially adsorbed casein, and possible interfacial activity enhancement, thereby identifying AprX-producing *Pseudomonas species* as a primary enzymatic threat to cream liqueur stability and suggesting that quality control strategies should focus on minimising psychrotrophic contamination in raw milk, while metalloprotease-specific inhibition approaches (e.g., zinc chelation using EDTA or phosphates) may be more effective than general protease inhibition.

It is important to clarify that the proteolytic risk identified in this study is not microbiological in nature: the alcoholic, low-water-activity environment of cream liqueur is intrinsically inhibitory to *Pseudomonas* and *Bacillus* growth, and the well-established two-year ambient shelf life of commercial cream liqueurs reflects this. The risk arises instead from the upstream stage-the cold storage of raw milk and cream-where psychrotrophic populations have ample opportunity to secrete heat-stable AprX and HhoA. Because these enzymes survive pasteurisation, even modest enzymatic loads carried into the bottled product can, over months to years of ambient storage, produce sufficient cumulative casein hydrolysis to compromise emulsion stability. The present experimental design was chosen to isolate this carry-over effect from any microbial activity in the finished product. By implication, the most effective control point for managing AprX-mediated cream liqueur destabilisation lies upstream of the liqueur formulation itself: minimising psychrotrophic load in raw milk and cream through stringent cold-chain management, refrigerated storage time limits, and microbiological monitoring at intake, rather than additional intervention in the bottled product.

Several limitations should be acknowledged; the complex cream liqueur matrix makes it challenging to isolate the specific factors responsible for attenuation of *Bacillus* protease activity, and future studies using simplified model systems with incremental addition of individual components would help elucidate these mechanistic bases; the azocasein standardisation approach, while providing a uniform measure of general proteolytic activity, may not fully capture differences in kinetics or specificity in complex matrices; the study employed a single commercial formulation, and variations in fat content, alcohol level, emulsifier type, and calcium content among brands may influence susceptibility to proteolytic destabilisation; and the accelerated storage conditions used here, while necessary for practical experimental feasibility, may not directly translate to shelf-life predictions under realistic contamination scenarios, underscoring the need for complementary long-term studies at lower, more physiologically representative enzyme concentrations.

5. Conclusions

This study provides the first systematic comparison of bacterial protease behaviour in cream liqueur versus conventional milk systems, revealing pronounced matrix-dependent effects with important implications for quality control. AprX from *Pseudomonas fluorescens* is the dominant enzymatic threat to cream liqueur stability, achieving extensive casein hydrolysis after 20 days, compared with lower levels for *B. cereus* HhoA. The hierarchy of protease efficacy was reversed between matrices: *B. cereus* HhoA > AprX in skim milk, but AprX ≫ *B. cereus* HhoA in cream liqueur, demonstrating that activity cannot be predicted from simpler dairy systems. AprX-induced destabilisation manifested as characteristic biphasic viscosity response, particle size broadening (Span >3.0), and substantial colour change (ΔE = 12.8)-indicative of κ-casein cleavage and subsequent peptide aggregation.

The cream liqueur matrix-particularly ethanol, fat, and altered casein organisation-decreased *Bacillus* protease activity by 45–66% relative to skim milk. These findings identify psychrotrophic *Pseudomonas* contamination as a primary enzymatic risk factor for cream liqueur quality. This work extends understanding of bacterial proteases beyond conventional dairy systems and provides a foundation for developing evidence-based quality control strategies for cream liqueurs and related dairy-based alcoholic beverages. Future research should elucidate the specific matrix factors responsible for differential protease activity and evaluate practical intervention strategies under commercial production conditions.

CRediT authorship contribution statement

Yuetong Chen: Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Aritra Sinha:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis. **Alan L. Kelly:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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