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THE EFFECT OF CONCENTRATION AND TIME VARIATIONS OF THE LACTOSE AND SKIM MILK AS INDUCER ON THE EXPRESSION OF THE RECOMBINANT PROTEIN A224L AFRICAN SWINE FEVER VIRUS

Pengaruh Variasi Konsentrasi dan Waktu Induksi Laktosa dan Susu Skim Terhadap Ekspresi Protein Rekombinan Protein A224L Virus *African Swine Fever*

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Abstract

The development of subunit vaccines for African Swine Fever Virus (ASFV) requires efficient recombinant protein production. One of the potential vaccine candidate proteins is A224L, which plays a key role in inducing host cell apoptosis. This study evaluated the effectiveness of IPTG, lactose, and skim milk as inducers for the expression of recombinant A224L protein fused with GST in the pGEX-2TK-A224L plasmid. The tested concentrations of lactose and skim milk were 1%, 2.5%, and 5%, with observation times of 5, 24, and 48 hours after induction. The expression of GST-A224L was detected using dot blot with anti-GST antibody. The density of the dot was quantified using ImageJ software. The results showed that the type of inducer had a significant effect on the expression intensity of the recombinant GST-A224L protein. In addition, inducer concentration variations produced significant differences in signal intensity. Meanwhile, longer induction times did not yield stronger signal intensity. The 5% skim milk for 24 hours of induction produced the strongest signal equivalent to IPTG for 48 hours. This condition is recommended for application in the production of the ASFV A224L recombinant protein.

Keywords: GST-A224L, IPTG, lactose, recombinant protein expression, skim milk

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Abstrak

Pengembangan vaksin subunit untuk *African Swine Fever Virus* (ASFV) memerlukan produksi protein rekombinan yang efisien, salah satu protein yang dapat menjadi kandidat vaksin adalah protein A224L yang berperan dalam penghambatan apoptosis sel inang. Penelitian ini mengevaluasi efektivitas IPTG, laktosa, dan susu skim sebagai induktor ekspresi protein rekombinan A224L yang difusikan dengan GST dalam plasmid pGEX-2TK-A224L. Konsentrasi laktosa dan susu skim yang diuji-coba adalah 1%, 2,5% dan 5%, waktu pengamatan adalah 5 jam, 24 jam, dan 48 jam. Hasil ekspresi GST-A224L dideteksi dengan metode *dot blot* menggunakan antibodi GST. Ketebalan dari tiap *dot* dikuantifikasi menggunakan perangkat lunak ImageJ. Hasil penelitian menunjukkan bahwa faktor jenis induktor berpengaruh sangat nyata terhadap intensitas ekspresi protein rekombinan GST-A224L. Disamping itu variasi konsentrasi induktor menghasilkan perbedaan intensitas sinyal yang nyata. Berikutnya, waktu induksi yang lebih lama tidak menghasilkan ketebalan sinyal yang paling tebal. Susu skim dengan konsentrasi 5% lama waktu 24 jam memiliki sinyal dengan kemiripan yang sebanding dengan IPTG lama 48 jam. Kondisi ini disarankan untuk diaplikasikan dalam produksi protein ASF-A224L.

Kata kunci: ekspresi protein rekombinan, GST-A224L, IPTG, laktosa, susu skim

INTRODUCTION

The development of a subunit vaccine against African swine fever virus (ASFV) requires effective and efficient production of recombinant proteins. One of the important ASFV genes is A224L, which encodes a protein involved in the modulation of apoptosis and host immune responses (Weng, 2024). The presence of the A224L gene enables the virus to inhibit apoptosis, thereby allowing viral replication to continue. ASFV is a complex DNA virus with a mortality rate that can reach 100% in pigs and causes substantial economic losses to the global swine industry and pork production sector (Dixon *et al.*, 2017; Zhang *et al.*, 2024). Therefore, the production of A224L protein as a subunit vaccine candidate represents a strategic approach for ASF control.

The Faculty of Veterinary Medicine has developed the pGEX-2TK-A224L plasmid, in which the A224L protein is expressed as a fusion protein with Glutathione S-Transferase (GST). The GST fusion system is expected to enhance the stability and solubility of the target protein while minimizing the formation of inclusion bodies during the expression process (Harper and Speicher, 2011). The success of recombinant protein expression is determined not only by plasmid construction but also by the type of inducer, its concentration, and the duration of induction. The most commonly used inducer is Isopropyl β-D-1-thiogalactopyranoside (IPTG), a non-metabolizable lactose analog that effectively activates the lac promoter. However, the use of IPTG has several limitations, including potential cellular toxicity at certain concentrations and induction periods, relatively high cost, and the risk of producing improperly folded proteins due to excessively rapid expression rates (Neubauer *et al.*, 1992). Alternative inducers such as lactose and skim milk have the potential to replace IPTG because they are more economical and may support cellular metabolism more effectively. Lactose serves as both a carbon source and an inducer, whereas the nutritional components of skim milk may help maintain the stability of the protein synthesis process.

Variations in inducer concentration and induction time are known to significantly influence the expression level, solubility, and quality of the resulting protein. Excessive inducer concentrations may impose a metabolic burden on cells, whereas suboptimal induction periods may reduce protein yield or solubility (Weber *et al.*, 2021). To date, no standardized protocol has been established to specifically define the optimal conditions for the use of lactose and

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skim milk as inducers in the expression of recombinant A224L protein. Therefore, this study aimed to compare the expression intensity of GST-A224L protein induced by IPTG, lactose, and skim milk, and to determine the optimal inducer concentration and induction time using the dot blot method as a rapid and efficient semi-quantitative analytical approach (Mishra, 2022).

MATERIALS AND METHODS

Research Object

The object of this study was recombinant A224L protein expressed in *Escherichia coli* BL21. The expression vector used was the pGEX-2TK plasmid carrying the A224L gene. Protein expression was induced using several types of inducers, namely IPTG, lactose, and skim milk at different concentrations. The resulting A224L protein was analyzed to evaluate expression levels and assess the potential of alternative inducers.

Research Design

This study employed an experimental approach using a completely randomized design (CRD) with a factorial arrangement. Four treatment groups were included: a negative control group consisting of *E. coli* BL21 cultured in TBG medium without the addition of an inducer; a positive control group consisting of *E. coli* BL21 carrying the pGEX-2TK-A224L plasmid and induced with 0.1 mM IPTG in TBG medium; a lactose treatment group consisting of *E. coli* BL21 carrying the pGEX-2TK-A224L plasmid and induced with lactose at concentrations of 1%, 2.5%, and 5% in TB medium; and a skim milk treatment group consisting of *E. coli* BL21 carrying the pGEX-2TK-A224L plasmid and induced with skim milk at concentrations of 1%, 2.5%, and 5% in TB medium.

All treatment groups were evaluated at three incubation periods, namely 5, 24, and 48 hours, with three replicates per treatment. Each treatment group was therefore assessed across nine combinations of inducer concentration and incubation time. A total of 27 samples were analyzed for each inducer type.

Preparation of Terrific Broth (TB) and Terrific Broth Glycerol (TBG) Media

TB medium (Invitrogen, USA) and TBG medium were prepared by dissolving 23.5 g of TB powder in 500 mL of sterile distilled water according to the manufacturer's instructions. For TBG preparation, 23.5 g of TB powder was dissolved in 500 mL of sterile distilled water, followed by the addition of 2 mL glycerol after the medium had been thoroughly mixed. The media were heated in a microwave oven until boiling, allowed to stand at room temperature for 30 minutes, and subsequently sterilized by autoclaving at 121°C for 15 minutes before use in bacterial culture.

Preparation of Lactose and Skim Milk Inducers

The inducers were prepared using two sterile 500-mL Schott Duran bottles. Lactose and skim milk stock solutions were prepared by dissolving 125 g of lactose powder or skim milk powder, respectively, in 500 mL of sterile distilled water. The solutions were heated in a microwave oven for 30 minutes and sterilized by autoclaving at 121°C for 15 minutes. Working concentrations of 1%, 2.5%, and 5% were subsequently prepared by dilution of the stock solutions.

A224L Culture

A224L cultures were prepared in 6 mL of TBG medium supplemented with 1% ampicillin and inoculated with 2 µL of *E. coli* BL21 carrying the pGEX-2TK plasmid. For the negative

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control, ampicillin was omitted. The cultures were incubated at 37°C for 48 hours with shaking at 120 rpm using an incubator shaker.

Induction Using Lactose and Skim Milk

The amplified bacterial cultures were centrifuged to obtain cell pellets, which were subsequently resuspended in TB medium according to the respective inducer treatments. Induction was performed using 0.1 mM IPTG as the positive control, while lactose and skim milk were used at final concentrations of 1%, 2.5%, and 5%. The volume of each inducer was determined using the dilution formula to achieve a final volume of 3 mL for each treatment.

The negative control consisted of 3 mL TBG medium. The positive control consisted of 2,970 µL TBG supplemented with 30 µL ampicillin and 30 µL IPTG. For the lactose treatments, 2,880 µL TB supplemented with 30 µL ampicillin and 120 µL lactose was used for the 1% concentration, 2,700 µL TB supplemented with 30 µL ampicillin and 300 µL lactose for the 2.5% concentration, and 2,400 µL TB supplemented with 30 µL ampicillin and 600 µL lactose for the 5% concentration. Similarly, the skim milk treatments consisted of 2,880 µL TB supplemented with 30 µL ampicillin and 120 µL skim milk for the 1% concentration, 2,700 µL TB supplemented with 30 µL ampicillin and 300 µL skim milk for the 2.5% concentration, and 2,400 µL TB supplemented with 30 µL ampicillin and 600 µL skim milk for the 5% concentration. The cultures were incubated, and 500 µL aliquots were collected at 5, 24, and 48 hours for subsequent analysis.

Confirmation of Protein Expression Intensity by Dot Blot

Expression of the recombinant GST-A224L protein was confirmed using the dot blot method. Samples were mixed with buffer and boiled at 100°C for 8 minutes before being spotted onto a nitrocellulose membrane. The membrane was blocked with skim milk solution in PBST (Phosphate-Buffered Saline with Tween) for 1 hour to prevent nonspecific binding. The membrane was then washed three times with PBST, each wash lasting 5 minutes.

Subsequently, the membrane was incubated for 1 hour with anti-GST primary antibody diluted 1:100 in PBST (40 µL anti-GST in 40 mL PBST), followed by three washes with PBST, each lasting 5 minutes. The membrane was then incubated with anti-mouse secondary antibody at the same dilution ratio for 1 hour and washed again three times for 5 minutes each. In the final step, the membrane was immersed in an alkaline phosphatase (AP) substrate solution to visualize protein expression signals. The substrate solution consisted of 500 µL reagent A, 500 µL reagent B, and 200 µL AP color reagent in a final volume of 48 mL PBST.

Evaluation of Dot Blot Signal Intensity Using ImageJ

Dot blot signal intensity was quantitatively analyzed using ImageJ software (NIH, USA). Dot blot membranes were photographed under uniform lighting conditions using a smartphone camera with consistent contrast settings. The images were converted to 8-bit grayscale format and analyzed using the Region of Interest (ROI) method to determine the intensity of each spot. Signal intensity was quantified using the integrated density parameter. The quantitative values obtained for each sample were normalized using the fold-change method by comparing the intensity value of each treatment with that of the control (treatment/control).

Data Analysis

Dot blot signal intensity data from all treatments were tabulated and statistically analyzed using IBM SPSS Statistics for Windows version 25. The Shapiro–Wilk test was used to assess data normality. The main analysis was performed using two-way analysis of variance (two-way ANOVA) with inducer concentration and induction time as factors to evaluate their main

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effects and interaction on recombinant GST-A224L protein expression levels. When significant differences were detected, Tukey's Honestly Significant Difference (Tukey HSD) post hoc test was applied for pairwise comparisons among treatments. Statistical significance was established at $\alpha = 0.05$.

RESULTS AND DISCUSSIONS

Results

Escherichia coli BL21 carrying the pGEX-2TK-A224L plasmid was successfully cultured prior to the induction process. The bacterial cultures exhibited normal growth and relatively uniform turbidity. A representative image of the A224L plasmid-bearing bacterial culture before induction is presented in Figure 1. Dot blot analysis revealed protein expression signals in the form of dots with varying intensities among treatments, indicating differences in A224L protein expression levels. Representative dot blot results for all treatments are presented in Figure 2.

The quantified dot blot intensities obtained using ImageJ software for recombinant GST-A224L protein expression at 5, 24, and 48 hours after induction with 0.1 mM IPTG, lactose (1%, 2.5%, and 5%), and skim milk (1%, 2.5%, and 5%) are presented in Table 1. The results of the Shapiro–Wilk normality test for recombinant GST-A224L protein expression values across different inducer concentrations and induction times are shown in Table 2. The normality test indicated that several treatment groups did not meet the assumption of normal distribution. Significant deviations from normality ($p < 0.05$) were observed in the lactose 1%, skim milk 2.5%, and skim milk 5% treatment groups.

The results of the two-way ANOVA performed on the mean dot blot intensity values, representing recombinant GST-A224L protein expression under different inducer concentrations and induction times, are presented in Table 3. The analysis demonstrated that inducer type, induction time, and the interaction between these factors had highly significant effects on protein expression levels ($p < 0.001$).

Post hoc analysis using Tukey's Honestly Significant Difference (Tukey HSD) test is presented in Table 4. Among the alternative inducers evaluated, the highest expression intensity was obtained with 5% skim milk, followed by 2.5% skim milk and 1% skim milk. Lactose induction resulted in significantly lower expression levels than those achieved with 1% and 2.5% skim milk ($p < 0.05$). The bar chart presented in Figure 3 provides a clearer visualization of the differences in mean GST-A224L protein expression intensity among treatments.

Discussion

This study explored the potential of lactose and skim milk as alternative inducers to IPTG in an *Escherichia coli* lac operon-based expression system, offering a more cost-effective approach with improved compatibility for cellular physiology. The Shapiro–Wilk normality test indicated that several datasets were not normally distributed in certain treatment groups (lactose 5%, skim milk 1%, and skim milk 2.5%), likely due to limited sample size and inherent biological variability in recombinant protein expression systems. Nevertheless, statistical analysis was continued using two-way ANOVA, as this method is robust to mild violations of normality in factorial designs with balanced replication and homogeneous variance, and it also enables evaluation of interactions between inducer type and induction time (Lantz, 2013).

The results demonstrated that induction using skim milk, particularly at a concentration of 5% with a 24-hour induction period, produced the highest expression intensity of GST-A224L compared to lactose. The lower expression observed with lactose induction is likely associated with limited utilization of lactose as an energy source in the pGEX-2TK plasmid system, where insertion of the target gene disrupts the lacZ open reading frame, preventing optimal lactose metabolism by the host

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cells (Harper and Speicher, 2008). This condition may lead to protein expression occurring without sufficient energy support and potentially increases metabolic stress, especially during prolonged induction periods (Kilikian *et al.*, 2000; Neubauer *et al.*, 1992).

The superiority of skim milk is attributed to its additional nutritional components, including proteins, peptides, and alternative carbohydrates, which serve as supplementary sources of carbon and nitrogen, thereby supporting cell growth and maintaining translational activity during induction (de Divitiis *et al.*, 2023; Rosano and Ceccarelli, 2014). Khani and Bagheri (2020) further reported that skim milk enables cells to maintain a more stable metabolic state, resulting in more consistent and efficient protein expression.

The fold change approach used in this study employed time-matched controls as the reference, defined as 1. Fold change values represent the relative change in protein expression levels under each treatment compared to the corresponding control at the same induction time. A fold change greater than 1 indicates an increase in expression relative to the control, suggesting effective induction under the given conditions. Conversely, a fold change of less than 1 indicates lower expression than the control, which does not imply absence of expression but rather insufficient induction above basal detectable levels (Hanel and Carlberg, 2022; Jiang *et al.*, 2025).

The lack of a significant effect of induction time suggests that GST-A224L expression reached an optimal level during the mid-induction phase (24 hours), while prolonged induction did not further enhance expression. This phenomenon supports the concept that overexpression of viral proteins may impose metabolic burden on the host translational machinery and trigger cellular stress responses when induction is extended excessively (Jürgen *et al.*, 2010; Neubauer and Hofmann, 1994). Overall, these findings support the development of skim milk-based induction protocols as an economical and sustainable alternative for recombinant protein production, particularly for viral target proteins such as A224L, which may be further explored as a potential ASF vaccine candidate.

CONCLUSION AND SUGGESTIONS

Conclusions

The type of inducer had a highly significant effect on the expression intensity of recombinant GST-A224L protein ($p < 0.001$), whereas variations in inducer concentration resulted in significant differences in signal intensity ($p < 0.05$). In contrast, prolonged induction time did not produce the strongest protein expression signal and had no significant effect on GST-A224L expression ($p > 0.05$).

Suggestions

A skim milk concentration of 5% is recommended for the production of recombinant ASFV A224L protein. Further studies investigating the effects of gene expression on metabolic stress are warranted to improve understanding of the underlying mechanisms involved. In addition, optimization of culture density through the assessment of optical density prior to induction may further enhance protein expression yield.

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Tables

Table 1. Densitometric intensity values of GST-A224L protein expression quantified using ImageJ in E. coli BL21 culture at 48 hours, and observed at 5, 24, and 48 hours post-induction with 0.1 mM IPTG, 1%, 2.5%, and 5% lactose, and 1%, 2.5%, and 5% skim milk.

Induction time	Replication	Inducer							
		Control	IPTG	Lactose			Skim Milk		
				1%	2.5%	5%	1%	2.5%	5%
5	1	1.0	8.9	1.0	0.8	1.2	9.0	8.9	9.2
	2	1.1	8.8	1.0	0.9	1.3	9.1	8.7	9.2
	3	1.0	7.8	1.0	0.8	1.2	8.9	8.8	9.1
	Mean	1.0	8.5	1.0	0.8	1.2	9.0	8.8	9.2
	SD	0.1	0.6	0.0	0.1	0.1	0.1	0.1	0.1
24	1	1.0	8.9	1.1	1.2	0.9	6.8	7.8	9.8
	2	0.7	7.5	1.1	1.2	0.8	6.8	7.9	9.9
	3	1.3	6.5	1.2	1.5	0.8	6.6	7.8	9.7
	Mean	1.0	7.6	1.1	1.3	0.8	6.7	7.8	9.8
	SD	0.3	1.2	0.1	0.2	0.1	0.1	0.1	0.1
48	1	0.7	10.2	1.1	1.2	2.1	6.0	3.4	9.1
	2	0.9	11.8	1.0	1.0	2.0	1.7	9.2	5.9
	3	1.4	11.2	1.1	1.4	2.2	5.8	2.5	8.9
	Mean	1.0	11.1	1.1	1.2	2.1	4.5	5.0	8.0
	SD	0.4	0.8	0.1	0.2	0.1	2.4	3.6	1.8

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Table 2. Shapiro–Wilk normality test results of GST-A224L protein expression levels across different treatment groups.

Treatment	Statistic	Significance (p-value)	Distribution
Negative control	0.930	0.486	Normal
IPTG 0.1 mM	0.959	0.785	Normal
Lactose 1%	0.805	0.024	Abnormal
Lactose 2.5%	0.897	0.235	Normal
Lactose 5%	0.851	0.077	Normal
Skim Milk 1%	0.847	0.069	Normal
Skim Milk 2.5%	0.731	0.003	Abnormal
Skim Milk 5%	0.661	0.000	Abnormal

Table 3. Two-way ANOVA results for recombinant GST-A224L protein expression intensity based on induction time and inducer concentration.

Source of variation	Type III Sum of Squares	Degrees of Freedom	Mean Square	F-value	Significance (p-value)
Inducer	887.724	7	126.818	40.195	0.000
Time	6.135	2	3.068	2.929	0.063
Time * Inducer	74.411	14	5.315	5.075	0.000
Error	50.273	48	1.047		
Total	2523.720	72			

Table 4. Post hoc Tukey HSD test results showing the effects of inducer concentration and induction time on recombinant GST-A224L protein expression intensity.

Treatment	Time	Concentration	Mean	Grouping Letters
Lactose	5 hours	2.5 %	0.8	a
Lactose	24 hours	5%	0.8	a
Lactose	5 hours	1%	1.0	a
Negative control	24 hours	-	1.0	a
Negative control	48 hours	-	1.0	a
Negative control	5 hours	-	1.0	a
Lactose	48 hours	1%	1.1	a
Lactose	48 hours	2.5%	1.2	a
Lactose	24 hours	1%	1.2	a
Lactose	5 hours	5 %	1.2	a
Lactose	24 hours	2.5%	1.3	a
Lactose	48 hours	5%	2.1	ab
Skim milk	48 hours	1%	4.5	bc
Skim milk	48 hours	2.5%	5.0	cd
Skim milk	24 hours	1%	6.7	cde
IPTG	24 hours	0.1mM	7.6	def
Skim milk	24 hours	2.5%	7.8	ef
Skim milk	48 hours	5%	8.0	ef
IPTG	5 hours	0.1 mM	8.5	efg
Skim milk	5 hours	2.5%	8.8	efg
Skim milk	5 hours	1%	9.0	efg
Skim milk	5 hours	5%	9.1	efg
Skim milk	24 hours	5%	9.8	fg
IPTG	48 hours	0.1 mM	11.1	g

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Figures

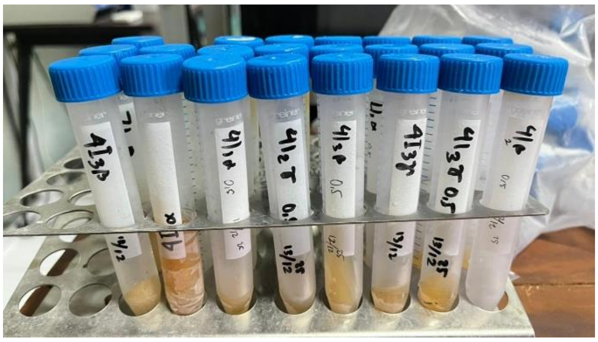

Figure 1. Escherichia coli BL21 culture harboring the A224L plasmid.

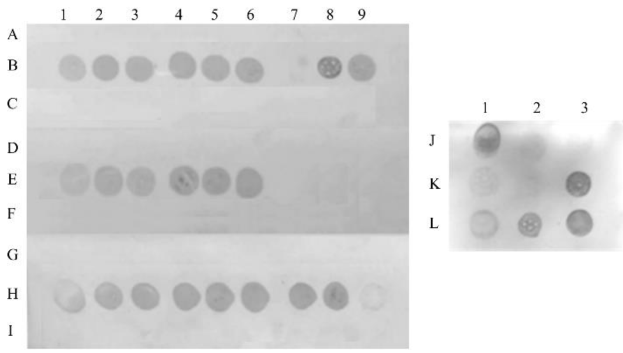

Figure 2. Dot blot analysis of recombinant GST-A224L protein expression. The left panel shows dot blot results for lactose (A, D, G), skim milk (B, E, H), and control (C, F, I) at three different time points: 5 hours (A–C), 24 hours (D–F), and 48 hours (G–I). The right panel shows IPTG (0.1 mM) induction at 5 hours (J), 24 hours (K), and 48 hours (L), each performed in triplicate. IPTG, lactose, and skim milk inductions were performed at different time points.

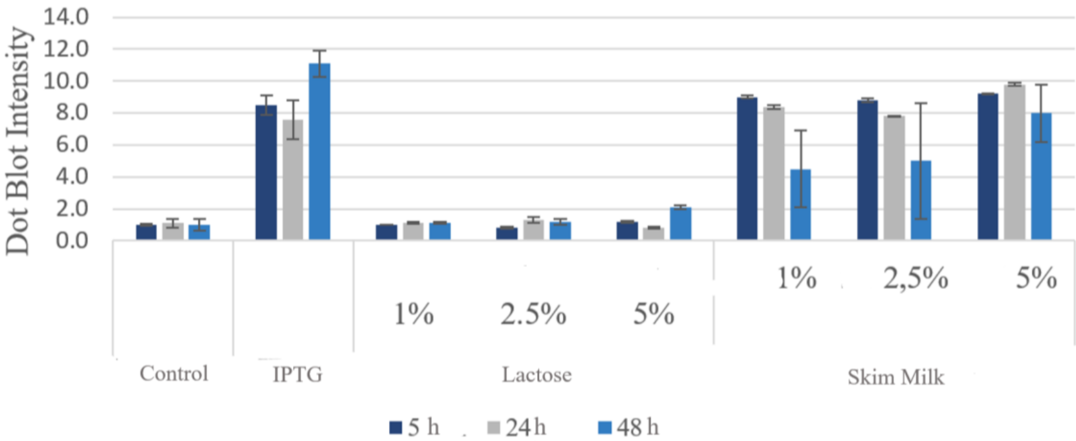

Figure 3. Mean densitometric intensity of dot blot signals measured using ImageJ under different inducer concentrations and induction times.

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