CCDC174 Gene Expression as a Molecular Marker of Bull Fertility

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SUMMARY

Conventional semen evaluation methods do not fully capture molecular differences among ejaculates with similar motility profiles. The CCDC174 gene has been associated with sperm motility and fertility in dairy bulls, but its predictive value across breeds under field classification systems remains unclear. This study evaluated whether CCDC174 expression differs between fertile and sub-fertile ejaculates and whether it predicts progressive motility. A total of 53 ejaculates from nine bulls of three dairy breeds (Friesian, Ayrshire, and Jersey) were analyzed at the National Artificial Insemination Centre, Tanzania. Ejaculates were classified as fertile or sub-fertile based on progressive motility (PR > 40% or ≤ 40%). CASA was used to assess sperm kinetics. CCDC174 expression was quantified using qPCR, with GAPDH as the reference gene. Associations between variables were analyzed using appropriate statistical tests, and a linear mixed-effects model was used to evaluate predictors of progressive motility. Most ejaculates (81.1%) were classified as fertile. CCDC174 expression differed significantly between fertile and sub-fertile groups. Progressive motility was associated with breed and gene expression in unadjusted analysis. In the mixed-effects model, CCDC174 expression and specific sperm kinetic parameters were significant predictors of progressive motility, while breed and season were not significant after adjustment. The study concludes that CCDC174 gene expression is associated with sperm progressive motility and independently predicts fertility-related parameters beyond conventional semen evaluation. These findings support its potential use as a complementary molecular marker in bull fertility assessment.

Keywords: Progressive motility, bull fertility, sperm mRNA, gene expression, artificial insemination.

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INTRODUCTION

The reproductive efficiency of cattle herds that depend on artificial insemination (AI) is shaped, to an outsized degree, by the fertility of individual sires. A single bull in a commercial AI programme may contribute genetic material to several thousand females each year, so that even a small deficit in his fertilising capacity cascades into extended calving intervals, elevated repeat-service rates, and compromised genetic progress across the herd (Butler et al., 2020; DeJarnette et al., 2004). Identifying and removing sub-fertile males from the donor pool before their semen enters the distribution chain is therefore a first-order objective in reproductive management, yet the laboratory

tools currently deployed for this purpose capture only part of the picture (Thundathil et al., 2016).

Routine semen evaluation relies on macroscopic and microscopic parameters: ejaculate volume, sperm concentration, total and progressive motility, morphological normality, and indices of membrane and acrosomal integrity (Jung et al., 2015; Zuidema et al., 2021). The introduction of computer-assisted sperm analysis (CASA) added objectivity and dimensional depth to these assessments. CASA digitises sperm tracks and extracts a suite of kinetic descriptors, among them curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN), straightness (STR), and wobble (WOB) (Kathiravan et al., 2011; Mortimer and Mortimer, 2012). Of these, progressive motility (PR) occupies a privileged position in operational semen quality control. PR measures the proportion of spermatozoa exhibiting rapid, forward-directed translocation, a behaviour required for traversing the female tract and penetrating the zona pellucida (Waberski et al., 2022). Field and controlled-trial data have repeatedly confirmed that PR correlates with conception outcome more tightly than most other single kinetic indices, and many AI centres use a PR threshold to accept or discard ejaculates for processing (Li et al., 2016; Kathiravan et al., 2011).

Where this approach runs into difficulty is that bulls whose CASA profiles are indistinguishable can nevertheless achieve discrepant pregnancy rates under identical management (Riveros et al., 2023; Gliozzi et al., 2017; Correa et al., 1997). This gap has fueled interest in molecular determinants of sperm function that operate below the resolution of kinetic analysis. Over the past two decades, a body of work has established that mammalian spermatozoa, though transcriptionally silent after nuclear condensation, retain a defined set of messenger RNAs carried forward from late spermatogenesis and epididymal transit (Santiago et al., 2022; Li and Zhou, 2012; Boerke et al., 2007). Certain of these transcripts participate in post-testicular remodeling, capacitation signaling, and early embryonic gene regulation once delivered to the oocyte (Caballero et al., 2011; Card et al., 2013; Indriastuti et al., 2022). Variation in the abundance of specific sperm-borne mRNAs has been linked to fertility differences in bulls, boars, and men, opening a path toward biomarker-assisted sire evaluation (Ugur et al., 2022; Parthipan et al., 2017; Gross et al., 2020).

One candidate that has surfaced from this line of work is the Coiled-Coil Domain Containing 174 (CCDC174) gene. Selvaraju et al. (2021), working with Holstein Friesian bulls in India, reported that CCDC174 expression correlated with both semen quality indices and field conception rates. At the protein level, CCDC174 is thought to contribute to axonemal assembly and to mitochondrial energy coupling in the sperm midpiece, both of which feed directly into flagellar beat generation (Selvaraju et al., 2021). These functional attributes lend biological plausibility to the gene's candidacy as a fertility-informative transcript. Three gaps, however, limit the translational value of the evidence as it stands. First, validation has been confined to one breed under temperate management; whether the association holds across genetically distinct populations managed in tropical environments is unknown. Second, the earlier classification of bulls relied on conception rate, a retrospective endpoint confounded by female factors, insemination technique, and herd management. A classification built on PR, which is measured at the point of semen processing, would be more operationally tractable and less exposed to extraneous variance. Third, the statistical treatment in existing reports has been limited to bivariate comparisons (t-tests, correlations) that neither account for the hierarchical structure of repeated ejaculates within bulls nor control for the joint influence of breed, season, and the full panel of CASA kinetics.

MATERIALS AND METHODS

Study Site and Animals

The work was conducted at the National Artificial Insemination Centre (NAIC), Arusha, Tanzania, the country's principal repository for frozen bovine semen. Nine mature breeding bulls representing three dairy breeds (Friesian, Ayrshire, and Jersey) were used. All bulls were housed under uniform management with ad libitum water access and a ration formulated for mature breeding males. Routine health screening, vaccination, and reproductive soundness examinations followed NAIC standard operating procedures.

Semen Collection

Ejaculates were collected using the artificial vagina (AV) method. Prior to each collection, the bull's preputial area was shaved, washed, and dried. Two false mounts preceded the collection mount. The AV was sterilised and pre-warmed to 42 to 45 degrees Celsius and fitted with a transparent collection tube. Immediately after ejaculation, each sample was inspected macroscopically for volume, colour, and contaminants. Samples meeting acceptance criteria were placed in a water bath at 37 degrees Celsius for no more than ten minutes before kinetic analysis. Collections spanned both wet and dry seasons to permit evaluation of seasonal effects.

Computer-Assisted Sperm Analysis

Kinetic parameters were assessed with a Sperm Class Analyser (Microptic, Spain). Where cryopreserved straws were analysed, they were thawed at 37 degrees Celsius for 30 seconds, diluted with an equal volume of pre-warmed Easy-Buffer B (IMV Technologies, L'Aigle, France), and incubated at 37 degrees Celsius for 10 minutes. A 10-microlitre aliquot was placed on a pre-warmed microscope slide and ten homogeneous fields were captured (Selvaraju et al., 2021). The CASA system recorded total motility, progressive motility (PR), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN = VSL/VCL), straightness (STR = VSL/VAP), and wobble (WOB = VAP/VCL). Each ejaculate was assigned to a fertile group (PR > 40%) or a sub-fertile group (PR of 40% or below) on the basis of established bovine motility thresholds (Kathiravan et al., 2011).

Selection of Samples for Gene Expression Analysis

From the broader pool of ejaculates collected at NAIC, 53 samples (22 Friesian, 16 Ayrshire, 15 Jersey) were selected for CCDC174 gene expression analysis. The selection encompassed both fertile and sub-fertile ejaculates across all three breeds and both seasons, to ensure sufficient representation for bivariate and multivariable modelling.

Spermatozoa RNA Extraction and Reverse Transcription

Spermatozoa were washed three times with cold phosphate-buffered saline and pelleted by centrifugation at 16,000 g at 4 degrees Celsius for 10 minutes, yielding pellets of approximately 10 to 40 million cells. Total RNA was isolated with the TransZol Up Plus RNA Kit (TransGen Biotech, ER501) with minor modifications. Sperm pellets were resuspended in TRIzol containing 3 microlitres of glycogen (20 micrograms per microlitre; Invitrogen), homogenised, and incubated at 65 degrees Celsius for 15 minutes with gentle agitation. RNA was separated with chloroform and precipitated from the aqueous phase using isopropanol. Pellets were washed twice with 70% ethanol, air-dried for 5 to 10 minutes, and dissolved in nuclease-free water. RNA quantity and quality were verified by spectrophotometry and Agilent Bioanalyser. Complementary DNA was synthesised with SuperScript IV reverse transcriptase (Thermo Scientific, USA) and stored at minus 20 degrees Celsius until quantitative PCR.

Quantitative PCR

Reactions were set up using Luna Universal One-Step Reaction Mix (New England Biolabs). Each 20-microlitre reaction contained 10 microlitres of Luna Universal One-Step Reaction Mix (2X), 1 microlitre of Luna WarmStart RT Enzyme Mix (20X), 0.8 microlitres each of forward and reverse primers (10 micromolar), 4.4 microlitres of nuclease-free water, and 3 microlitres of RNA template. Two genes were targeted: the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the candidate fertility gene CCDC174. GAPDH primers were: forward CTGAGGACCAGGTTGTCTCCTG, reverse CCCTGTTGCTGTAGCCAAATTC. CCDC174 primers were: forward GGGTACTGGTCGAAGAGGCA, reverse CCTGGCTGCTCAGAGACTCA. Cycling comprised initial denaturation at 95 degrees Celsius for 60 seconds, 45 cycles of 95 degrees Celsius for 10 seconds and 60 degrees Celsius for 30 seconds, and a default melt-curve analysis (95 degrees Celsius for 15 seconds). Cycle threshold (CT) values were recorded using 7500 Software v2.

Selection of Housekeeping Gene and Expression Classification

GAPDH was chosen as the internal reference on the basis of its documented expression stability in bovine spermatozoa (Parthipan et al., 2017; Selvaraju et al., 2021). Relative CCDC174 mRNA expression was estimated using the delta-delta CT approach (Livak and Schmittgen, 2001). For the purposes of bivariate association testing, samples were dichotomised into high-expression and low-expression categories on the basis of their CCDC174 CT values. In the multivariable analysis, CCDC174 CT was retained as a continuous variable to preserve the dose-response gradient.

Statistical Analysis

Analyses were carried out with IBM SPSS Statistics (Version 20) and SAS (PROC MIXED). Descriptive statistics summarised the frequency distributions of breed, season, CT gene expression category, and PR classification. Given the small expected cell counts in several cross-tabulations, Fisher's exact test was used rather than the Pearson chi-square to evaluate bivariate associations between PR classification (fertile versus sub-fertile) and each categorical predictor (breed, season, CT expression category). For bivariate analysis, CCDC174 CT values were categorized into high- and low-expression groups based on their relative distribution within the dataset, whereas CT values were treated as a continuous variable in the multivariable mixed-effects model.

A linear mixed-effects model was fitted to quantify the independent contribution of CCDC174 CT expression to PR while accounting for within-bull clustering and the simultaneous influence of other covariates. Progressive motility served as the continuous response. Bull identity was entered as a random intercept to absorb unmeasured between-bull heterogeneity arising from factors such as age, libido, or testicular capacity. Fixed effects comprised breed (Friesian, Ayrshire, Jersey; Jersey as reference), season (dry, wet; wet as reference), CCDC174 CT value (continuous), and the full set of CASA kinetic parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF). This specification permitted the gene expression effect to be estimated net of confounding by breed, seasonality, and sperm biophysical characteristics. Statistical significance was set at $P < 0.05$. Because several kinetic parameters (in particular VCL, VSL, VAP, and the ratio-derived measures LIN, STR, WOB) share mathematical dependencies, the model estimates should be interpreted with awareness that partial collinearity may inflate standard errors for individual kinetic covariates; this is addressed further in the limitations.

Ethical Approval

The study received approval from the Institutional Animal Ethical Committee (IAEC). All procedures involving animals adhered to established guidelines for ethical handling and sampling.

RESULTS

Descriptive Characteristics of the Study Sample

Table 1 summarises the 53 ejaculates included in the gene expression analysis. Friesian bulls contributed the largest share (22 samples, 41.51%), followed by Ayrshire (16, 30.19%) and Jersey (15, 28.30%). Twenty-four samples (45.28%) were collected in the dry season and 29 (54.72%) in the wet season. When classified by CCDC174 CT values, 39 samples (73.58%) fell into the high-expression category and 14 (26.42%) into the low-expression category (Figure 1). Applying the 40% PR threshold, 43 ejaculates (81.13%) were classified as fertile and 10 (18.87%) as sub-fertile.

Table 1. Descriptive characteristics of the 53 ejaculate samples.

Variable	(n)	%
Breed		
Friesian	22	41.51
Ayrshire	16	30.19
Jersey	15	28.30
Season		
Dry	24	45.28
Wet	29	54.72
CT Gene Expression		
Low expression	14	26.42
High expression	39	73.58
Progressive Motility		
Sub-fertile (PR ≤ 40%)	10	18.87
Fertile (PR > 40%)	43	81.13

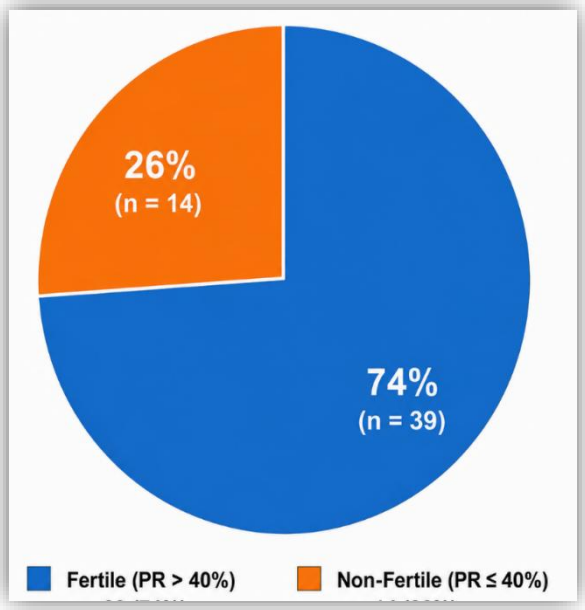

Figure 1. *Distribution of CCDC174 CT gene expression categories among 53 bull ejaculate samples (high expression: 73.58%; low expression: 26.4*

Bivariate Associations with Fertility Classification

Fisher's exact test was applied to each of the three categorical predictors in turn (Table 2).

Breed. A significant association was detected between breed and PR classification (P = 0.0020). Every Jersey ejaculate (15 of 15, 100.00%) exceeded the 40% PR threshold. Among Ayrshire samples, 15 of 16 (93.75%) met the fertile criterion. Friesian bulls showed the highest sub-fertility prevalence: 9 of 22 ejaculates (40.91%) fell at or below 40% PR.

Season. No association was found between season and fertility status (P = 0.9999). The proportions of fertile ejaculates during the dry season (19 of 24, 79.17%) and the wet season (24 of 29, 82.76%) were virtually identical.

CT Gene Expression. A significant association was observed (P = 0.0075). Among high-expression ejaculates, 35 of 39 (89.74%) were fertile and only 4 (10.26%) sub-fertile. Among low-expression ejaculates, the ratio shifted markedly: 8 of 14 (57.14%) were fertile and 6 (42.86%) sub-fertile. Expressed as odds, sub-fertility was roughly 6.6 times more likely in the low-expression group than in the high-expression group (odds ratio: [6/8]/[4/35] = 6.56).

Linear Mixed-Effects Model

Table 3 presents the fixed-effect solutions. After simultaneous adjustment, breed was no longer significantly associated with PR. Estimates for Friesian (4.94, SE = 5.13, P = 0.342) and Ayrshire (5.30, SE = 4.42, P = 0.238), both relative to the Jersey reference, indicated positive deviations that did not reach significance. Season likewise lost its effect (dry versus wet: -0.24, SE = 3.91, P = 0.950).

CT gene expression retained statistical significance (estimate = -1.06, SE = 0.52, P = 0.048). Because CT values are inversely related to initial template concentration in quantitative PCR, the negative sign indicates that each unit rise in CT (reflecting lower CCDC174 mRNA abundance) was associated with a 1.06 percentage-point reduction in progressive motility, after all other variables had been accounted for.

Among the eight kinetic covariates, only beat cross frequency achieved independent significance (estimate = 2.34, SE = 0.98, P = 0.022). The remaining parameters did not reach the 0.05 threshold: VCL (P = 0.276), VSL (P = 0.719), VAP

(P = 0.398), LIN (P = 0.199), STR (P = 0.180), WOB (P = 0.336), ALH (P = 0.625).

Table 2. Association of bull characteristics with progressive motility classification (Fisher's exact test).

Variable	Sub-fertile N (%)	Fertile N (%)	P-value
Breed			**0.0020***
Friesian	9 (40.91)	13 (59.09)	
Ayrshire	1 (6.25)	15 (93.75)	
Jersey	0 (0.00)	15 (100.00)	
Season			0.9999
Dry	5 (20.83)	19 (79.17)	
Wet	5 (17.24)	24 (82.76)	
CT Gene Expression			**0.0075***
Low expression	6 (42.86)	8 (57.14)	
High expression	4 (10.26)	35 (89.74)	

** Significant at P < 0.05.*

Table 3. Fixed-effect solutions from the linear mixed-effects model for progressive motility.

Variable	Estimate	SE	DF	t Value	P-value
Intercept	-489.66	429.37	40	-1.14	0.261
Breed					
Friesian	4.94	5.13	40	0.96	0.342
Ayrshire	5.30	4.42	40	1.20	0.238
Jersey (ref.)	0	.	.	.	.
Season					
Dry	-0.24	3.91	40	-0.06	0.950
Wet (ref.)	0	.	.	.	.
CT Gene Expression	**-1.06**	0.52	40	-2.04	**0.048***
VCL	-0.65	0.59	40	-1.10	0.276
VSL	-0.81	2.22	40	-0.36	0.719
VAP	2.20	2.58	40	0.85	0.398
LIN	-13.76	10.53	40	-1.31	0.199
STR	6.85	5.02	40	1.36	0.180
WOB	9.33	9.59	40	0.97	0.336
ALH	5.44	11.04	40	0.49	0.625
BCF	**2.34**	0.98	40	2.39	**0.022***

*Random effect: bull identity (random intercept). Reference categories: Jersey (breed), Wet (season). * P < 0.05. SE = standard error; DF = denominator degrees of freedom.*

DISCUSSION

The principal finding of this study is that CCDC174 CT gene expression is an independent predictor of progressive motility in bovine spermatozoa, remaining significant after adjustment for breed, season, and CASA kinetic variables in a linear mixed-effects model with bull as a random intercept. This extends previous work (Selvaraju et al., 2021) by confirming the association in a multi-breed population under tropical conditions and by demonstrating that the relationship persists even after controlling for a comprehensive set of sperm kinetic parameters.

Most ejaculates in this study exceeded the 40% progressive motility threshold (81.13%), indicating generally good semen quality at NAIC. However, the presence of a sub-fertile fraction (18.87%) highlights the limitations of relying solely on conventional motility thresholds for semen evaluation. This aligns with prior observations that such thresholds are useful screening tools but insufficient to fully capture functional fertility variation (Li et al., 2016). From a practical standpoint, even a relatively small proportion of compromised ejaculates is relevant in artificial insemination systems where a single ejaculate may generate large numbers of insemination doses.

Breed differences were evident in the unadjusted analysis, with Jersey bulls showing uniformly fertile classification and Friesian bulls exhibiting higher sub-fertility rates. Similar breed-associated variation in sperm quality traits has been reported previously (Morrell et al., 2017; Christensen et al., 2011). However, these differences were no longer significant in the adjusted model, suggesting that breed effects are largely explained by underlying variation in sperm kinetics and gene expression rather than representing independent biological determinants. This indicates that individual ejaculate-level assessment may be more informative than breed-based assumptions in semen evaluation.

Seasonal effects were not observed at either the bivariate or multivariable level, suggesting that current management practices at the AI centre effectively buffer environmental variation. While the lack of recorded microclimatic data limits finer interpretation, the consistency across seasons is operationally encouraging and suggests that seasonal climate fluctuations are not a major determinant of progressive motility in this setting.

The central result of this study is the consistent association between CCDC174 expression and progressive motility. Bulls with lower CCDC174 transcript levels showed reduced motility in both bivariate and multivariable analyses, and the effect remained significant after adjustment for all CASA variables. This indicates that CCDC174 expression captures a dimension of sperm quality not fully reflected by standard kinetic measures. Importantly, its retention in the model suggests that molecular profiling provides additional discriminatory power beyond conventional semen analysis.

Among CASA variables, only beat cross frequency (BCF) was independently associated with progressive motility in the adjusted model. This reinforces the role of BCF as a robust indicator of flagellar beating efficiency and sperm progression. The simultaneous significance of both BCF and CCDC174 suggests that kinetic measurements and gene expression may represent complementary levels of sperm function, with the former reflecting immediate motility characteristics and the latter capturing broader cellular preparedness influencing motility performance.

The use of a linear mixed-effects model strengthens the validity of these findings by accounting for repeated measurements within bulls and allowing simultaneous adjustment for multiple covariates. This approach reduces bias from unobserved bull-level effects and provides more reliable estimates than simpler bivariate analyses. It also clarifies that some apparent associations at the unadjusted level, particularly breed effects, are not independent predictors when molecular and kinetic variables are considered together.

From a practical perspective, incorporating CCDC174 expression into semen evaluation protocols could improve the precision of sire selection in artificial insemination programs. While CASA-based thresholds remain useful, they do not fully capture underlying molecular variation associated with motility differences. A combined approach integrating gene expression with kinetic assessment may therefore enhance prediction of semen quality and reproductive performance.

Given the scale of artificial insemination programs, even modest improvements in predictive accuracy could translate into meaningful gains in fertility outcomes at the population level.

GAPDH showed stable expression across samples and was suitable as a reference gene in this dataset, consistent with previous reports in bovine spermatozoa (Parthipan et al., 2017). Nevertheless, validation of additional reference genes in future studies would strengthen confidence in normalization strategies, particularly in larger and more diverse populations.

Several limitations should be acknowledged. The relatively small sample size and single-centre design limit generalisability, and the ratio of predictors to observations raises the possibility of model overfitting. In addition, some CASA variables are mathematically interrelated, which may introduce collinearity and reduce the ability to detect independent effects of individual kinetic parameters. The use of progressive motility as a fertility proxy rather than direct pregnancy outcomes is another limitation, although it remains a widely accepted indicator of semen quality. Finally, the dichotomisation of gene expression for part of the analysis reduces informational resolution, and future studies should rely primarily on continuous modelling approaches.

Future work should validate these findings in larger, multi-site cohorts and across different cattle genotypes. Linking CCDC174 expression directly to field fertility outcomes would be particularly valuable. In addition, multi-gene or multi-omics approaches may provide a more comprehensive understanding of sperm function than single-marker models.

5. CONCLUSION

CCDC174 mRNA abundance in bull spermatozoa independently predicts progressive motility, and it does so after accounting for breed, season, and the full suite of CASA kinetic parameters in a mixed-effects model. High CCDC174 expression was strongly associated with fertile ejaculate classification, while low expression was enriched among sub-fertile samples. Beat cross frequency was the only kinetic descriptor that shared independent predictive significance with gene expression, suggesting complementary biological information. The attenuation of breed effects from bivariate significance to non-significance in the multivariable model exposes the limitation of breed-level generalisations relative to ejaculate-level molecular assessment for fertility prediction. On this evidence, CCDC174 expression shows strong potential as a biomarker for bull fertility and may be considered a promising candidate for inclusion in future sire evaluation frameworks at AI centers, with the important caveat that validation against field conception rates and replication in larger, multi-site cohorts should precede operational implementation. Multi-gene panels and multi-omics integration offer the most promising path toward a practical molecular screening protocol for bull fertility assessment.

ETHICAL APPROVAL

The study received approval from the Institutional Animal Ethical Committee (IAEC). All procedures involving animals complied with established ethical guidelines.

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Disclosure Statement

The authors declare no conflict of interest.

Author Contributions

J.D. Mbuma: conceptualization, methodology, formal analysis, investigation, data curation, and original draft preparation. G.M. Msalya and I.P. Kashoma: supervision, critical review, and manuscript revision. H.E. Chimeledya: data collection and analysis support.

Data Availability

Data supporting these findings are available from the corresponding author upon reasonable request.

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