

**UNIVERSIDADE ESTADUAL PAULISTA “JULIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS DE BOTUCATU**

**FREQUÊNCIAS DAS VARIANTES ASSOCIADAS AOS GENES
CALPAIN E *CALPASTATIN* EM BOVINOS DA RAÇA BRAHMAN
NO BRASIL**

LUKAS GARRIDO ALBERTINO

**BOTUCATU – SÃO PAULO
SETEMBRO DE 2024**

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LUKAS GARRIDO ALBERTINO

Tese apresentada junto ao programa
de Pós Graduação em Medicina
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Doutor.

Orientador: Prof.º Ass. Dr. José Paes
de Oliveira-Filho

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IMPACTO POTENCIAL DESTA PESQUISA: Este é o primeiro estudo a avaliar quatro marcadores genéticos (*CAPN1-316*, *CAPN1-4751*, *CAPN3* e *CAST*) para maciez de carne, um importante componente de palatabilidade e aceitação do consumidor, na raça Brahman no Brasil, e também a criar um protocolo com técnica (*ARMS-PCR*) considerada de baixo custo para determinar genótipos sem necessidade de sequenciamento genético. Os resultados encontrados neste estudo podem ajudar produtores e criadores na seleção de animais com alelos favoráveis para maciez de carne usando um método de baixo custo, visando obter produtos com melhores características de carne e maiores lucros para produção.

POTENTIAL IMPACT OF THIS RESEARCH: This is the first study to evaluate four gene markers (*CAPN1-316*, *CAPN1-4751*, *CAPN3*, and *CAST*) for meat tenderness, an important component of palatability and acceptance of consumers, in Brahman cattle in Brazil, and also to create a protocol with a technique (*ARMS-PCR*) considered a simple low-cost method to determine genotypes with no need for genetic sequencing. The results observed in this study may help producers and breeders to select animals with favorable meat tenderness alleles, using a simple and low-cost method, aiming to obtain products with a better meat characteristic and more profits for production.

Nome do Autor: Lukas Garrido Albertino

Título: FREQUÊNCIAS DAS VARIANTES ASSOCIADAS AOS GENES *CALPAIN* E *CALPASTATIN* EM BOVINOS DA RAÇA BRAHMAN NO BRASIL

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LISTA DE ABREVIações

A – Adenina

ABBA – *American Brahman Breeders Association*

ABCZ – Associação Brasileira dos Criadores de Zebu

ABIEC – Associação Brasileira das Indústrias Exportadoras de Carne

ABPA – Associação Brasileira de Proteína Animal

ARMS-PCR – Amplification refractory mutation system

AS-PCR – Allele specific PCR

ACBB – Associação dos Criadores de Brahman do Brasil

C - Citosina

CAPN1 – Gene μ -calpain; gene micro-calpain

CAPN2 – Gene m-calpain

CAPN3 – Gene calpain3

CAST – Gene *calpastatin*; gene calpastatina

DNA – ácido desoxirribonucleico

EUA – Estados Unidos da América

G - Guanina

GCC – Alanina

GGC – Glicina

HRM-PCR – High resolution melting PCR

IBGE – Instituto Brasileiro de Geografia e Estatística

kg – Quilogramas

PCR – Reação em cadeia da polimerase

PIB – Produto Interno Bruto

qRT-PCR – Real time reverse transcriptase PCR

RFLP-PCR – Restriction fragment lenght polymorphism

SNP – Polimorfismo de nucleotídeo único

T - Timina

TEC – Toneladas equivalente carcaça

UTR – Região não codificante

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RESUMO

A maciez da carne é considerada como importante componente de palatabilidade e aceitação pelo consumidor, e certos biomarcadores podem ser utilizados para identificar animais com melhor maciez, como aqueles associados ao sistema calpaína-calpastatina. Neste estudo, as frequências alélicas dos biomarcadores *CAPN1-316*, *CAPN1-4751*, *CAPN3* e *CAST* foram analisados em reprodutores e matrizes da raça Brahman no Brasil. Um total de 108 amostras de DNA de Brahman (n=54 reprodutores e n=54 matrizes) foram utilizadas neste estudo. As amostras foram submetidas a PCR e sequenciamento direto de Sanger para determinar o genótipo dos animais e comparar as sequências e eletroferogramas com *amplicons* previamente determinados para a técnica *ARMS-PCR*. O biomarcador *CAST* foi o mais prevalente neste estudo, com 48% (52/108 amostras) classificadas como homozigoto A/A. Consequentemente, um protocolo e *primers* para a técnica de *ARMS-PCR* foram desenvolvidos e validados para a variante *CAST*. A técnica de *ARMS-PCR* para o gene *CAST* mostrou-se 100% eficiente, relativamente simples e de baixo custo na determinação do genótipo do animal. Esses resultados podem auxiliar produtores e criadores a obter produtos com melhor características de carne, contribuindo para melhor aceitação pelo consumidor e ganhos para produção.

Palavras chave: *ARMS-PCR*, *CAPN1*, *CAPN3*, *CAST*, estudo de prevalência,

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ABSTRACT

Meat tenderness is an important component of palatability and acceptance for the consumer, and certain genetic markers may be used to identify cattle with better meat tenderness, such as those in the *calpain-calpastatin* system. In this study, the allele frequencies of the gene markers *CAPN1*-316, *CAPN1*-4751, *CAPN3*, and *CAST* were assessed in Brazilian Brahman bulls and cows. A total of 108 Brahman DNA samples (n=54 bulls and n=54 cows) were assessed in this study. The samples were submitted to PCR and Sanger direct sequencing to determine the animals' genotype and to compare the sequencing and electropherograms obtained with the previous amplicons of the *ARMS*-PCR technique. The gene marker *CAST* was the most prevalent, with 48% (52/108 samples) classified as homozygous A/A in this study. Consequently, a protocol and primers for the *ARMS*-PCR technique were devised and validated for the *CAST* variant. The *ARMS*-PCR technique for the *CAST* gene has proven to be 100% effective, relatively simple, cost-effective, and user-friendly in determining the animal's genotype. These results may help producers and breeders obtain products with better meat characteristics, contributing to better acceptance by the consumer and profits for production.

Keywords: *ARMS*-PCR, *CAPN1*, *CAPN3*, *CAST*, prevalence study

CAPÍTULO I

1. Introdução

O Brasil é detentor do segundo maior rebanho bovino do mundo, considerado como o segundo maior produtor de carne bovina e maior exportador mundial de carne bovina. Dentre as raças de corte criadas em nosso país, podemos destacar os Brahman, animais zebuínos, originários dos Estados Unidos, caracterizados por sua adaptabilidade ambiental, habilidade materna, longevidade, produção eficiente de carne e docilidade. Com sua genética presente em mais de 70 países e apresentando ótimos resultados, os Brahman são criados de forma pura ou em cruzamentos com gado europeu, tanto para produção de carne quanto para dupla aptidão (leite e carne). A melhora do crescimento e ganho de peso do animal e melhora das características e qualidade de carcaça são o principal objetivo de criadores e pesquisadores no gado de corte, sendo a maciez da carne um fator importante e crítico para aceitação e satisfação do consumidor, estando associada a fatores como a degradação de proteínas miofibrilares post-mortem por ação do sistema proteolítico cálcio-dependente. A degradação das proteínas miofibrilares post-mortem é resultado da ação de diversas enzimas proteolíticas autólogas, dentre elas o complexo enzimático dos genes calpaína e calpastatina. Diversos polimorfismos de nucleotídeo único nos genes calpaína e calpastatina foram descritos e associados a maciez de carne em estudos mundialmente e no Brasil com raças bovinas de origem zebuína, taurina e cruzamentos, entretanto nenhum deles foi realizado no Brasil utilizando a raça Brahman como foco de estudo. A maioria dos estudos publicados faz uso da reação em cadeia da polimerase associada a enzimas de restrição e sequenciamento genético para determinação do genótipo do animal analisado, mas poucos fazem uso da técnica de *ARMS-PCR*, um método simples, de baixo custo e alta especificidade para determinar polimorfismos sem a necessidade de sequenciamento genético do animal e que vem sendo utilizada em mapeamentos de diversas mutações genéticas.

2. Revisão de literatura

2.1. A pecuária do Brasil

De acordo com dados fornecidos pelo IBGE (Instituto Brasileiro de Geografia e Estatística), até o ano de 2022 o rebanho bovino era constituído por 234 milhões de cabeças (IBGE, 2023), o que representa 12,18% do rebanho mundial e é considerado como o segundo maior rebanho bovino do mundo, de acordo com a Associação Brasileira das Indústrias Exportadoras de Carne (ABIEC, 2023).

O Brasil também ocupa o segundo lugar mundial na produção de carne bovina, com 10,79 milhões de toneladas equivalente carcaça (TEC) (14,34% da produção mundial), e o primeiro lugar mundial na exportação de carne bovina, com 3,02 milhões de TEC (27,7% das exportações mundiais) (ABIEC, 2023).

Dos dez milhões de TEC produzidos, sete milhões foram destinadas ao mercado interno nacional, com um consumo per capita de 23,54 kg/ano (ABIEC, 2023), o segundo maior consumo de carne no país, perdendo apenas para a carne de frango, com consumo per capita de 45,2 kg/ano (ABPA, 2023).

A indústria de carne bovina representou 41,6% do PIB (produto interno bruto) total do agronegócio no ano de 2022, onde movimentou R\$ 1,02 trilhões de reais, sendo esta a maior participação do setor na geração de riqueza nacional (ABIEC, 2023).

2.2. A raça Brahman

Formada nos EUA (Estados Unidos da América) no final do século XIX e início do XX (ACBB, 2023), a raça Brahman é fruto do cruzamento de zebuínos importados da Índia e do Brasil, especialmente animais das raças Nelore, Guzará, Gir, Khrisna Valley e Indubrasil (ABCZ, 2023; ACBB, 2023), e possui características marcáveis associadas a adaptabilidade ambiental, habilidade materna, longevidade, produção eficiente de carne e docilidade (ABBA, 2023; ACBB, 2023).

A ABBA (*American Brahman Breeders Association*; tradução livre para “Associação Americana de Criadores de Brahman”), foi fundada no ano de 1924, no Texas, e onde o nome “Brahman”, que significa “um novo ciclo”, foi proposto para os animais da raça (ABBA, 2023).

No Brasil, a introdução da raça americana sofreu resistência pelos criadores brasileiros de zebuínos, considerada como *“um animal mestiço e não uma raça pura, que retrocederia o trabalho desenvolvido pela Associação Brasileira dos Criadores de Zebu”* [...] *“no fato dele decretar a asfixia de uma importante parcela da moderna pecuária do zebu brasileiro, sobretudo Tabapuã e Nelore Mocha”* [...] por ser *“o melhor zebu dos Estados Unidos, mas terá sempre muito pouca utilidade nos trópicos”* [...] e pelo *“gado Brahman ter perdido sua rusticidade. Tornou-se um animal aparentemente adequado ao estágio atual da pecuária norte-americana ... exagerando no preparo de animais para exposições e feiras, engordando excessivamente”* (LEMOS, 2006).

Após muita insistência pela ABBA, e de criadores brasileiros de Brahman nos EUA (LEMOS, 2006), a chegada oficial de bovinos da raça é datada de 1994 (LEMOS, 2006; ABCZ, 2023; ACBB, 2023), com a importação de 57 animais oriundos dos EUA, Argentina e Paraguai (LEMOS, 2006), passando a ser registrada pela Associação Brasileira dos Criadores de Zebu (LEMOS, 2006; ABCZ, 2023).

Doze anos após sua introdução oficial no país e abertura dos livros da raça, os Brahman demonstraram que podiam caminhar muito bem como instrumento de produzir mais carne com qualidade (LEMOS, 2006).

Atualmente, a genética do Brahman está presente em mais de 70 países, apresentando ótimos resultados (ABBA, 2023; ABCZ, 2023; ACBB, 2023), pois é uma das raças mais utilizadas em regiões tropicais e subtropicais, criados de forma pura ou em cruzamentos com gado europeu, tanto para produção de carne quanto para dupla aptidão (leite e carne) (HERNÁNDEZ et al., 2016). Até o ano de 2020, a raça contava com 224.481 animais registrados no Brasil, com peso médio de desmama de 198 kg para machos e 186 kg para fêmeas, e sobreano de 330 kg para machos e 282 kg para fêmeas criados a pasto (ABCZ, 2023).

Os Brahman são a segunda raça de corte que mais exporta sêmen no Brasil, ficando atrás apenas do Nelore. No ano de 2022, de acordo com o Anuário 2023 da Revista DBO, saíram do país quase 66 mil doses de sêmen para países da América Latina e Ásia, e a comercialização de animais movimentou R\$ 9,4 milhões, com a média de venda no valor de R\$ 15.823 (DBO, 2023).

2.3. Sistema proteolítico cálcio-dependente e a maciez da carne

Um dos principais objetivos dos pesquisadores e criadores no gado de corte, é a melhora do crescimento e ganho de peso do animal e melhora das características e qualidade de carcaça (RAZA et al., 2020), sendo a maciez da carne um fator importante e crítico para aceitação e satisfação do consumidor (CASAS et al., 2006; ALLAIS et al., 2011; CHUNG, SHING e CHUNG, 2014).

A qualidade da carne, durante o processo de crescimento e ganho de peso do animal, não é rotineiramente avaliada (ALLAIS et al., 2011) e a obtenção de dados fenotípicos antes do abate é dificultosa (WHITE et al., 2005), visto que diversos fatores ambientais e genéticos (RAZA et al., 2020) e atributos associados a carne, como maciez, retenção de água, coloração e outros (LEAL-GUTIÉRREZ et al., 2018) influenciam a mesma.

Como citado anteriormente, a maciez da carne é um fator importante e crítico para aceitação e satisfação do consumidor (CASAS et al., 2006; ALLAIS et al., 2011; CHUNG, SHING e CHUNG, 2014), e depende principalmente da quantidade de tecido conjuntivo, degradação de proteínas miofibrilares post-mortem e presença de gordura intramuscular na carne (LEAL-GUTIÉRREZ et al., 2018).

A degradação das proteínas miofibrilares post-mortem é resultado da ação de diversas enzimas proteolíticas autólogas (NATTRASS et al., 2014; LEAL-GUTIÉRREZ et al., 2018), dentre as quais podemos destacar aquelas associadas ao gene *calpain* (gene *calpaína*), um grupo de proteases intracelulares cálcio-dependentes e que tem papel central na proteólise e maciez da carne post-mortem (BA e INHO, 2013). Este grupo de enzimas é representado por três genes, sendo eles o gene μ -calpain (micro-calpain;

CAPN1), que possui principal ação proteolítica e influência sobre a maciez da carne (GEESINK et al., 2006; KEMP et al., 2010; BA e INHO, 2013), e os genes m-calpain (*CAPN2*) e calpain3 (*CAPN3*) (BA e INHO, 2013; NATTRASS et al., 2014; LEAL-GUTIÉRREZ et al., 2018), que, de acordo com estudos, não estão envolvidos no processo de maciez post-mortem (GEESINK et al., 2006; KEMP et al., 2010), apesar de discordâncias a respeito do gene *CAPN3* (BARENDSE et al., 2008).

O papel fisiológico em organismos vivos dos *calpain* ainda não está completamente elucidado, com diversas controvérsias a respeito de sua ação (BA e INHO, 2013). Entretanto, já foi descrito que possuem um papel importante na remodelação citoesquelética e na sinalização, apoptose e sobrevivência celular (STORR et al., 2011; BA e INHO, 2013; LEAL-GUTIÉRREZ et al., 2018).

Em contraste à ação dos genes *calpain*, o gene *calpastatin* (gene calpastatina) atua como inibidor endógeno sobre estes genes, responsável por modular a proteólise das fibras musculares post-mortem (KOOHMARAIE e GEESINK, 2006; NATTRASS et al., 2014; BLECHA et al., 2019) e atuar na viabilidade e proliferação celular *in vivo* (VAN BA, BANGDULA VENKATA e INHO, 2015; LEAL-GUTIÉRREZ et al., 2018).

A ativação do complexo enzimático calpaína e calpastatina se inicia após o abate do animal, visto que o post-mortem estimula e aumenta a atividade do gene *calpain*, e está diretamente envolvido com o processo de maciez da carne durante a fase de maturação (BLECHA et al., 2019), que consiste na estocagem de cortes cárneos, por 14 a 21 dias, em temperatura acima do ponto de congelamento da carne, ou seja, entre 0° e 4°C.

Apesar de muitos polimorfismos (SNP) nos genes *CAPN1*, *CAPN3* e *CAST* terem sido descritos e associados a maciez de carne em diversas raças bovinas, nenhuma variante funcional foi identificada (LEAL-GUTIÉRREZ et al., 2018). Na raça Brahman, dentre os SNP estudados podemos destacar o *CAPN1*-316_g.5709C>G (PAGE et al., 2002), *CAPN1*-4751_g.6545C>T (WHITE et al., 2005), *CAPN3*_c.1538+225G>T (BARENDSE et al., 2008) e *CAST*_c.2832A>G (Barendse [2002], citado por CAFE et al., 2010ab).

2.3.1. CAPN1-316_g.5709C>G

Descrito por Page e colaboradores (2002), o SNP CAPN1-316_g.5709C>G é caracterizado pela substituição de uma citosina (C) por uma guanina (G), modificando uma alanina (GCC) por uma glicina (GGC), no éxon 9 no cromossomo 29 (PAGE et al., 2002). Essa substituição, pode provocar alterações funcionais na atividade proteolítica do μ -calpain com consequentes variações post-mortem de degradação miofibrilar (PAGE et al., 2002; CURI et al., 2010)

A presença do alelo C/C no SNP CAPN1-316_g.5709C>G é considerado como favorável para a maciez da carne (PAGE et al., 2002; WHITE et al., 2005; VAN EENENNAAM et al., 2007; CAFE et al., 2010ab; CURI et al., 2010), havendo uma diminuição na força de cisalhamento de 0,35 kg (PAGE et al., 2002) a 0,55 kg (WHITE et al., 2005), sendo este SNP considerado um bom marcador para animais de raças taurinas e cruzamentos (CURI et al., 2010), visto que a frequência alélica nos Brahman encontrada foi de 0,18% (CAFE et al., 2010b; GREENWOOD et al., 2013) a 1% (ROJAS, HERRERA e ZULUAGA, 2022).

Além dos Brahman, este SNP foi analisado mundialmente e em diversas raças, com variação de dados entre raças e estudos. Tais informações estão descritas na Tabela 1.

Tabela 1. Descrição dos dados observados em estudos realizados com o SNP CAPN1-316_g.5709C>G.

RAÇA	C/C	G/G	REFERÊNCIA	GENOTIPAGEM	PAÍS
Angus	47%	11%	Morris et al., 2006	Sistema MassArray	Nova Zelândia
	5%	59%	Van Eenennaam et al., 2007	Não informado	EUA
	10%	40%	Miquel et al., 2009	Enzimas polimerase	Argentina
	0%	81,8%	Soria et al., 2010	RFLP-PCR	Argentina

Continuação – Tabela 1

RAÇA	C/C	G/G	REFERÊNCIA	GENOTIPAGEM	PAÍS
Angus	21,73%	30,43%	Nattrass et al., 2014	<i>qRT</i> -PCR	Austrália
<i>Aosta Black Pied cattle</i>	0%	100%	Lisa e Stasio, 2009	<i>RFLP</i> -PCR	Itália
<i>Aosta Red Pied cattle</i>	6%	94%	Lisa e Stasio, 2009	<i>RFLP</i> -PCR	Itália
Belgian Blue	18%	82%	Lisa e Stasio, 2009	<i>RFLP</i> -PCR	Itália
Blonde d'Aquitaine	7%	93%	Lisa e Stasio, 2009	<i>RFLP</i> -PCR	Itália
	0,31%	91,72%	Allais et al., 2011	Enzimas polimerase	França
Braford	20%	30%	Ledesma, Coria e Palma, 2021	Não informado	Argentina
Brahman	0%	97,4%	Casas et al., 2005	Sistema MassArray	EUA
	0%	96%	Van Eenennaam et al., 2007	Não informado	EUA
			Cafe et al., 2010a		
			Robinson et al., 2012		
	0,29%	87,24%	Greenwood et al., 2013	<i>qRT</i> -PCR	Austrália
			Nattrass et al., 2014		

Continuação – Tabela 1

RAÇA	C/C	G/G	REFERÊNCIA	GENOTIPAGEM	PAÍS
Brahman	0,18%	90,44%	Cafe et al., 2010b	Enzimas polimerase	Austrália
			Greenwood et al., 2013		
	0%	100%	Soria et al., 2010	<i>RFLP</i> -PCR	Argentina
	0%	55%	Bosques et al., 2015	Não informado	Porto Rico
	0%	92,2%	Rojas et al., 2017	<i>HRM</i> -PCR	Colômbia
	0%	88,24%	Ardıçlı et al., 2019	<i>RFLP</i> -PCR	Turquia
	0%	100%	Pereira et al., 2022	<i>ARMS</i> -PCR	Bolívia
	1%	93%	Rojas, Herrera e Zuluaga, 2022	<i>HRM</i> -PCR	Colômbia
Brangus	2%	67%	Van Eenennaam et al., 2007	Não informado	EUA
	3,38%	61%	Miquel et al., 2009	Enzimas polimerase	Argentina
	0%	57%	Curi et al., 2010	<i>ARMS</i> -PCR	Brasil
	7,28%	53,44%	Mazzuco et al., 2010	Enzimas polimerase	Argentina
	4,7%	67,4%	Soria et al., 2010	<i>RFLP</i> -PCR	Argentina
Canchim	0%	82%	Curi et al., 2010	<i>ARMS</i> -PCR	Brasil
Charbray	0%	55%	Bosques et al., 2015	Não informado	Porto Rico
Charolês	0,36%	83,21%	Allais et al., 2011	Enzimas polimerase	França

Continuação – Tabela 1

RAÇA	C/C	G/G	REFERÊNCIA	GENOTIPAGEM	PAÍS
Charolês	1%	63%	Mejía et al., 2012	ACRS-PCR	México
	26%	53%	Avilés et al., 2013	Sequenciamento genético	Espanha
	4,86%	64,5%	Rincón et al., 2015	Sequenciamento genético	México
	0%	14,29%	Ardiçli et al., 2019	RFLP-PCR	Turquia
Creolle cattle	5,59%	55,94%	Pereira et al., 2015	ARMS-PCR	Colômbia
	25%	24%	Estevez et al., 2019	AS-PCR	Argentina
	3,7%	58,9%	Pereira et al., 2022	ARMS-PCR	Bolívia
Hanwoo	13,3%	35%	Chun, Shin e Chung, 2014	Enzimas polimerase	Coréia do Sul
Hereford	3%	56%	Morris et al., 2006	Sistema MassArray	Nova Zelândia
	4%	56%	Eenennaam et al., 2007	Não informado	EUA
	1%	86%	Melucci et al., 2012	RFLP-PCR e Enzimas polimerase	Argentina
	0%	27,59%	Ardiçli et al., 2019	RFLP-PCR	Turquia
Holstein	19%	81%	Lisa e Stasio, 2009	RFLP-PCR	Itália
	6,50%	51,25%	Ardicli et al., 2017a	RFLP-PCR	Turquia

Continuação – Tabela 1

RAÇA	C/C	G/G	REFERÊNCIA	GENOTIPAGEM	PAÍS
Limousin	7,17%	52,26%	Allais et al., 2011	Enzimas polimerase	França
	26%	62%	Avilés et al., 2013	Sequenciamento genético	Espanha
	0%	50%	Ardicli et al., 2019	<i>RFLP</i> -PCR	Turquia
Nelore	0%	92%	Curi et al., 2010	<i>ARMS</i> -PCR	Brasil
	0%	98,4%	Pinto et al., 2010	Enzimas polimerase	Brasil
	9,2%	88,5%	Pereira et al., 2022	<i>ARMS</i> -PCR	Bolívia
Parda Alpina	0%	86%	Curi et al., 2010	<i>ARMS</i> -PCR	Brasil
Piemontês	3%	85%	Lisa e Stasio, 2009	<i>RFLP</i> -PCR	Itália
<i>Retinta cattle</i>	59%	90%	Avilés et al., 2013	Sequenciamento genético	Espanha
<i>Romosinuano cattle</i>	2%	68%	Martínez-Rocha et al., 2023	Sequenciamento genético	México
Senepol	7%	55%	Bosques et al., 2015	Não informado	Porto Rico
Simental	0%	100%	Pereira et al., 2015	Enzimas polimerase	Brasil
	14,81%	85,19%	Ardicli et al., 2017b	<i>RFLP</i> -PCR	Turquia
<i>Turkish Grey cattle</i>	1,2%	78,9%	Kök et al., 2017	<i>ARMS</i> -PCR	Turquia
	0,3%	88%	Kök e Atalay, 2018	<i>ARMS</i> -PCR	Turquia

Continuação – Tabela 1

RAÇA	C/C	G/G	REFERÊNCIA	GENOTIPAGEM	PAÍS
Cruzamentos	34,78%	75,95%	Morris et al., 2006	Sistema MassArray	Nova Zelândia
	13,09%	34,18%	Corva et al., 2007	RFLP-PCR	Argentina
	4%	58%	Van Eennaamm et al., 2007	Não informado	EUA
	5,1%	78%	Frynlick et al., 2009	Sistema MassArray	África
	0%	100%	Curi et al., 2010	ARMS-PCR	Brasil
	6%	57%	Gruber et al., 2011	Não informado	EUA
	2%	74%	Rubio Lozano et al., 2016	Sequenciamento genético	México
	3%	86%	Desgarennés-Alcalá et al., 2017	Enzimas polimerase	México
	0%	33,33%	Ardiçli et al., 2019	RFLP-PCR	Turquia

No Brasil, três estudos associados ao SNP *CAPN1*-316_g.5709C>G foram realizados, um por Curi e colaboradores (2010), que utilizaram animais da raça Nelore, Brangus, Canchim, Parda Alpina e cruzamentos Angus x Nelore e Rubia Gallega x Nelore; um por Pinto e colaboradores (2010) e outro por Pereira e colaboradores (2015), que utilizaram respectivamente animais da raça Nelore e Simental. Nos três estudos, não foi encontrado nenhum animal alelo C/C para este SNP (CURI et al., 2010; PINTO et al., 2010; PEREIRA et al., 2015).

2.3.2. CAPN1-4751_g.6545C>T

O SNP CAPN1-4751_g.6545C>T é definido pela substituição de uma citosina (C) por uma timina (T), no íntron 17 no cromossomo 29 (WHITE et al., 2005).

Assim como no SNP descrito por Page e colaboradores (2002), a presença do alelo C/C no SNP CAPN1-4751_g.6545C>T é considerada como favorável para maciez da carne (WHITE et al., 2005; VAN EENENNAAM et al., 2007; CAFE et al., 2010ab), com uma diminuição na força de cisalhamento de 0,5 kg, ou seja, quando comparados animais Brahman alelo T/T (homozigotos) com animais Brahman alelo C/T (heterozigotos), há influência positiva na maciez da carne, sendo considerado como um excelente marcador na raça (WHITE et al., 2005). A frequência alélica observada do alelo C/C em bovinos da raça Brahman foi de 1% (VAN EENENNAAM et al., 2007) a 28,9% (ROJAS et al., 2017).

A descrição dos dados observados em estudos e das raças analisadas acerca do SNP CAPN1-4751_g.6545C>T estão descritas na Tabela 2.

Tabela 2. Descrição dos dados observados em estudos realizados com o SNP CAPN1-4751_g.6545C>T.

RAÇA	C/C	T/T	REFERÊNCIA	GENOTIPAGEM	PAÍS
	21%	26%	Van Eenennaam et al., 2007	Não informado	EUA
	36,4%	0%	Soria et al., 2010	<i>RFLP</i> -PCR	Argentina
Angus	66,66%	1,44%	Nattrass et al., 2014	<i>qRT</i> -PCR	Austrália
	17%	38%	Bosques et al., 2015	Não informado	Porto Rico
	45,84%	8,33%	Blecha et al., 2019	Enzimas polimerase	Brasil

Continuação – Tabela 2

RAÇA	C/C	T/T	REFERÊNCIA	GENOTIPAGEM	PAÍS
<i>Aosta Black Pied cattle</i>	38%	62%	Lisa e Stasio, 2009	<i>RFLP</i> -PCR	Itália
<i>Aosta Red Pied cattle</i>	39%	61%	Lisa e Stasio, 2009	<i>RFLP</i> -PCR	Itália
Belgian Blue	59%	41%	Lisa e Stasio, 2009	<i>RFLP</i> -PCR	Itália
Blonde d'Aquitaine	32%	62%	Lisa e Stasio, 2009	<i>RFLP</i> -PCR	Itália
Braford	43%	10%	Ledesma, Coria e Palma, 2021	Não informado	Argentina
Brahman	0%	75,39%	White et al., 2005	Sistema MassArray	EUA
	0%	80,85%	Casas et al., 2006	Sistema MassArray	EUA
	1%	88%	Van Eenennaam et al., 2007	Não informado	EUA
			Cafe et al., 2010a		
	6,82%	52,81%	Robinson et al., 2012	<i>qRT</i> -PCR	Austrália
			Greenwood et al., 2013		
			Nattrass et al., 2014		
			Cafe et al., 2010b		
	6,55%	62,5%	Greenwood et al., 2013	Enizmas polimerase	Austrália

Continuação – Tabela 2

RAÇA	C/C	T/T	REFERÊNCIA	GENOTIPAGEM	PAÍS
Brahman	0%	90%	Soria et al., 2010	<i>RFLP</i> -PCR	Argentina
	17%	38%	Bosques et al., 2015	Não informado	Porto Rico
	28,9%	60,6%	Rojas et al., 2017	<i>HRM</i> -PCR	Colômbia
	15,4%	82,7%	Pereira et al., 2022	<i>ARMS</i> -PCR	Bolívia
	20%	66%	Rojas, Herrera e Zuluaga, 2022	<i>HRM</i> -PCR	Colômbia
Brangus			Van		
	29%	27%	Eenennaam et al., 2007	Não informado	EUA
	10,5%	26,3%	Curi et al., 2009	<i>ARMS</i> -PCR	Brasil
	25,5%	23,48%	Mazzuco et al., 2010	Enzimas polimerase	Argentina
	23,3%	13,9%	Soria et al., 2010	<i>RFLP</i> -PCR	Argentina
Bonsmara	13,64%	4,54%	Blecha et al., 2019	Enzima polimerase	Brasil
Canchim	7,3%	36,6%	Curi et al., 2009	<i>ARMS</i> -PCR	Brasil
Caracu	53,85%	7,69%	Blecha et al., 2019	Enzima polimerase	Brasil
Charbray	17%	38%	Bosques et al., 2015	Não informado	Porto Rico
Charolês	19%	41%	Mejía et al., 2012	<i>ACRS</i> -PCR	México
	17%	38%	Bosques et al., 2015	Não informado	Porto Rico
	20,89%	29,20%	Rincón et al., 2015	Sequenciamento genético	México

Continuação – Tabela 2

RAÇA	C/C	T/T	REFERÊNCIA	GENOTIPAGEM	PAÍS
<i>Creolle cattle</i>	51,74%	6,29%	Pereira et al., 2015	ARMS-PCR	Colômbia
	55,8%	4,4%	Pereira et al., 2022	ARMS-PCR	Bolívia
Hanwoo	64,3%	4,9%	Chun, Shin e Chung, 2014	Enzimas polimerase	Coréia do Sul
Hereford	4%	71%	Van Eenennaam et al., 2007	Não informado	EUA
Holstein	81%	19%	Lisa e Stasio, 2009	RFLP-PCR	Itália
Nelore	0%	79%	Curi et al., 2009	ARMS-PCR	Brasil
	3,2%	67,5%	Pinto et al., 2010	Enzimas polimerase	Brasil
	0%	76,92%	Blecha et al., 2019	Enzimas polimerase	Brasil
	2,2%	81,1%	Pereira et al., 2022	ARMS-PCR	Bolívia
Parda Alpina	6,7%	60%	Curi et al., 2009	ARMS-PCR	Brasil
Piemontês	53%	47%	Lisa e Stasio, 2009	RFLP-PCR	Itália
<i>Romosinuano cattle</i>	21%	22%	Martínez-Rocha et al., 2023	Sequenciamento genético	México
Senepol	17%	38%	Bosques et al., 2015	Não informado	Porto Rico
	37,50%	16,67%	Blecha et al., 2019	Enzimas polimerase	Brasil

Continuação – Tabela 2

RAÇA	C/C	T/T	REFERÊNCIA	GENOTIPAGEM	PAÍS
Simental	26%	42%	Pereira et al., 2015	Enzimas polimerase	Brasil
<i>Turkish Grey cattle</i>	32%	18,9%	Kök et al., 2017	ARMS-PCR	Turquia
	17%	34	Kök e Atalay, 2018	ARMS-PCR	Turquia
Cruzamentos	36,83%	15,04%	White et al., 2005	Sistema MassArray	EUA
	36,99%	15,10%	Casas et al., 2006	Sistema MassArray	EUA
	19%	27%	Van Eenennaam et al., 2007	Não informado	EUA
	2,70%	59,45%	Curi et al., 2009	ARMS-PCR	Brasil
	17%	49,2%	Frynlick et al., 2009	Sistema MassArray	África
	38%	18%	Gruber et al., 2011	Não informado	EUA
	17%	38%	Bosques et al., 2015	Não informado	Porto Rico
	13%	42%	Rubio Lozano et al., 2016	Sequenciamento genético	México

Curi e colaboradores (2009), Pinto e colaboradores (2010), Pereira e colaboradores (2015) e Blecha e colaboradores (2019), foram responsáveis pelos estudos realizados no Brasil a respeito do SNP CAPN1-4751_g.6545C>T, utilizando as raças Angus (BLECHA et al., 2019), Brangus (CURI et al., 2009), Bonsmara (BLECHA et al., 2019), Canchim (CURI et al., 2009), Caracu (BLECHA et al., 2019), Nelore (CURI et al., 2009; PINTO et al.,

2010; BLECHA et al., 2019), Senepol (BLECHA et al., 2019), Simental (PEREIRA et al., 2015) e cruzamentos Angus x Nelore e Rubia Gallega x Nelore (CURI et al., 2009). A frequência do alelo favorável C/C observada nestes estudos foi de 53,85% na raça Caracu (BLECHA et al., 2019) e 3,2% na raça Nelore (PINTO et al., 2010).

2.3.3. *CAPN3_c.1538+225G>T*

Barendse e colaboradores (2008), descreveram o SNP associado ao gene *CAPN3*, caracterizado pela substituição de uma guanina (G) por uma timina (T), na posição c.1538+225G>T, do íntron 12 no cromossomo 10 (BARENDSE et al., 2008).

De acordo com estudos na raça Brahman, a presença do alelo G/G no SNP *CAPN3_c.1538+225G>T* é considerado favorável para a maciez da carne (BARENDSE et al., 2008; CAFE et al., 2010ab), com uma diminuição da força de cisalhamento de 0,14 kg (BARENDSE et al., 2008), entretanto há controvérsias a respeito da ação e efeitos do *CAPN3* sobre a maciez da carne (CAFE et al., 2010ab). A frequência alélica do alelo G/G observada nos Brahman foi de 27,03% (BARENDSE et al., 2008) a 37,68% (CAFE et al., 2010a; ROBINSON et al., 2012; GREENWOOD et al., 2013; NATTRASS et al., 2014), sendo este considerado como um SNP mais presente em raças zebuínas e cruzamentos do que em raças taurinas (BARENDSE et al., 2008).

A descrição dos dados observados em estudos e das raças analisadas acerca do SNP *CAPN3_c.1538+225G>T* estão descritas na Tabela 3. Até presente momento, nenhum estudo a respeito do SNP *CAPN3_c.1538+225G>T* foi realizado no Brasil.

Tabela 3. Descrição dos dados observados em estudos realizados com o SNP *CAPN3*_c.1538+225G>T.

RAÇA	G/G	T/T	REFERÊNCIA	GENOTIPAGEM	PAÍS
Angus	100%	0%	Nattrass et al., 2014	<i>qRT</i> -PCR	Austrália
Belmont Red	85,29%	0,38%	Barendse et al., 2008	Enzimas polimerase	Austrália
Brahman	27,03%	24,10%	Barendse et al., 2008	Enzimas polimerase	Austrália
			Cafe et al., 2010a		
			Robinson et al., 2012		
	37,68%	38,87%	Greenwood et al., 2013	<i>qRT</i> -PCR	Austrália
			Nattrass et al., 2014		
Brahman	27,40%	27,34%	Cafe et al., 2010b	Enzimas polimerase	Austrália
			Greenwood et al., 2013		
	34,56%	61,72%	Cafe et al., 2011	Não informado	Austrália
Santa Gertrudis	17%	38%	Bosques et al., 2015	Não informado	Porto Rico

Estudos a respeito do *CAPN3* também foram realizados em ovinos (ILIAN, BEKHIT e BICKERSTAFFE, 2004; KNIGHT et al., 2012; FANG et al., 2013; IBRAHIM et al., 2019; MEIRA et al., 2020; BÍLGEN et al., 2023; PURUHITA et al., 2023), suínos (GANDOLFI et al., 2011), aves (FELÍCIO et al., 2013; PIÓRKOWSKA et al., 2014) e em iaques (LI et al., 2013).

2.3.4. CAST_c.2832A>G

Barendse (2002), citado por Cafe colaboradores (2010ab), descreveu o SNP associado ao gene calpastatina, caracterizado pela substituição de uma adenina (A) por uma guanina (G), na posição c.2832A>G, na região 3'UTR (região não codificante), do éxon 30 no cromossomo 7 (CAFE et al., 2010ab; LI et al., 2010; BLECHA et al., 2019).

Nos Brahman, a presença do alelo A/A é considerada como favorável para a maciez da carne (CAFE et al., 2010ab), tendo efeito na força de cisalhamento de 0,47 kg (CASAS et al., 2006). A frequência do alelo favorável A/A em bovinos da raça Brahman foi de 30,38% (BARENDSE et al., 2008) a 52% (ROJAS, HERRERA E ZULUAGA, 2022), de acordo com estudos.

A descrição dos dados observados em estudos e das raças analisadas acerca do SNP CAST_c.2832A>G estão descritas na Tabela 4.

Tabela 4. Descrição dos dados observados em estudos realizados com o SNP CAST_c.2832A>G.

RAÇA	A/A	G/G	REFERÊNCIA	GENOTIPAGEM	PAÍS
Angus	89%	0%	Morris et al., 2006	Sistema MassArray	Nova Zelândia
	54,6%	24,2%	Li et al., 2010	RFLP-PCR	China
	100%	0%	Nattrass et al., 2014	qRT-PCR	Austrália
	88%	0%	Blecha et al., 2019	Enzimas polimerase	Brasil
Belmont Red	48,03%	3,54%	Barendse et al., 2008	Enzimas polimerase	Austrália
Blonde d'Aquitaine	58,49%	4,63%	Allais et al., 2011	Enzimas polimerase	França
Bonsmara	100%	0%	Blecha et al., 2019	Enzimas polimerase	Brasil

Continuação – Tabela 4

RAÇA	G/G	T/T	REFERÊNCIA	GENOTIPAGEM	PAÍS
Brahman	30,38%	18,55%	Barendse et al., 2008	Enzimas polimerase	Austrália
	44,21%	34,71%	Cafe et al., 2010a	<i>qRT</i> -PCR	Austrália
			Robinson et al., 2012		
			Greenwood et al., 2013		
	37,19%	19,41%	Natrass et al., 2014	Enizmas polimerase	Austrália
			Cafe et al., 2010b		
			Greenwood et al., 2013		
48,14%	45,67%	Cafe et al., 2011	Não informado	Austrália	
40%	23%	Rojas et al., 2017	<i>HRM</i> -PCR	Colômbia	
52%	20%	Rojas, Herrera e Zuluaga, 2022	<i>HRM</i> -PCR	Colômbia	
Brangus	68,4%	0%	Curi et al., 2009	<i>RFLP</i> -PCR	Brasil
Canchim	48,8%	9,7%	Curi et al., 2009	<i>RFLP</i> -PCR	Brasil
Caracu	56%	20%	Blecha et al., 2019	Enzimas polimerase	Brasil
Charolês	67,45%%	3,19%	Allais et al., 2011	Enzimas polimerase	França
	74,23%	3,24%	Rincón et al., 2015	Sequenciamento genético	México

Continuação – Tabela 4

RAÇA	G/G	T/T	REFERÊNCIA	GENOTIPAGEM	PAÍS
<i>Creolle cattle</i>	9%	11%	Estevez et al., 2019	AS-PCR	Argentina
Hereford	72%	3%	Morris et al., 2006	Sistema MassArray	Nova Zelândia
	55,6%	11,1%	Li et al., 2010	RFLP-PCR	China
<i>Jinnan cattle</i>	58,8%	23,5%	Li et al., 2010	RFLP-PCR	China
Limousin	67,30%	1,84%	Allais et al., 2011	Enzimas polimerase	França
<i>Luxi cattle</i>	40%	13,3%	Li et al., 2010	RFLP-PCR	China
Nelore	34,2%	22,8%	Curi et al., 2009	RFLP-PCR	Brasil
	16,85%	27,53%	Tizioto et al., 2014	Sequenciamento genético	Brasil
	30,77%	11,54%	Blecha et al., 2019	Enzimas polimerase	Brasil
Parda Alpina	46,7%	0%	Curi et al., 2009	RFLP-PCR	Brasil
<i>Qinchuan cattle</i>	50%	0%	Li et al., 2010	RFLP-PCR	China
<i>Romosinuano cattle</i>	11%	41%	Martínez-Rocha et al., 2023	Sequenciamento genético	México
Santa Gertrudis	41,55%	7,62%	Barendse et al., 2008	Enzimas polimerase	Austrália
Senepol	64%	12%	Blecha et al., 2019	Enzimas polimerase	Brasil
Simental	88%	0%	Pereira et al., 2015	Enzimas polimerase	Brasil

Continuação – Tabela 4

RAÇA	A/A	G/G	REFERÊNCIA	GENOTIPAGEM	PAÍS
Cruzamentos	99%	0%	Morris et al., 2006	Sistema MassArray	Nova Zelândia
	72,07%	0%	Curi et al., 2009	RFLP-PCR	Brasil
	94,1%	0%	Frynlick et al., 2009	Sistema MassArray	África
	54,78%	7,82%	Li et al., 2010	RFLP-PCR	China
	76%	0%	Gruber et al., 2011	Não informado	EUA

O SNP *CAST_c.2832A>G* foi estudado e analisado no Brasil por Curi e colaboradores (2009), Tizioto e colaboradores (2014), Pereira e colaboradores (2015) e Blecha e colaboradores (2019), com as raças Angus (BLECHA et al., 2019), Bonsmara (BLECHA et al., 2019), Brangus (CURI et al., 2009), Braunvieh (CURI et al., 2009), Canchim (CURI et al., 2009), Caracu (BLECHA et al., 2019), Nelore (CURI et al., 2009; TIZIOTO et al., 2014; BLECHA et al., 2019), Senepol (BLECHA et al., 2019), Simental (PEREIRA et al., 2015) e cruzamentos Angus x Nelore e Rubia Gallega x Nelore (CURI et al., 2009), onde foi observada a frequência do alelo A/A de 16,85% (TIZIOTO et al., 2014) a 100% (BLECHA et al., 2019) nas raças utilizadas para os estudos.

2.4. Determinação dos genótipos e a técnica de Amplification refractory mutation system (ARMS-PCR)

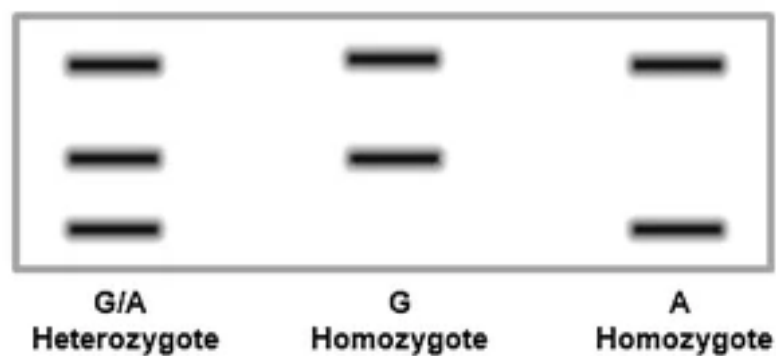
Nos estudos com a raça Brahman, diversas técnicas foram utilizadas para determinação dos genótipos dos animais analisados, dentre as quais podemos destacar: sistema MassArray (CASAS et al., 2005; WHITE et al., 2005; CASAS et al., 2006), enzimas polimerase (BARENDSE et al., 2008; CAFE et al., 2010b; GREENWOOD et al., 2013), qRT-PCR (*real time reverse transcriptase PCR*) (CAFE et al., 2010a; ROBINSON et al., 2012;

GREENWOOD et al., 2013; NATTRASS et al., 2014), HRM-PCR (*high resolution melting PCR*) (ROJAS et al., 2017; ROJAS, HERRERA e ZULUAGA, 2022), e RFLP-PCR (*restriction fragment length polymorphism PCR*) (SORIA et al., 2010; ARDIÇLI et al., 2019)

A técnica de ARMS-PCR é um método simples e de baixo custo para determinar SNP sem a necessidade de sequenciamento genético do animal (MEDRANO e OLIVEIRA, 2014). Possui uma alta especificidade na amplificação de produtos em uma única reação e vem sendo utilizada em mapeamentos de diversas mutações genéticas e na detecção do vírus causador da hepatite B em seres humanos (KUMAR et al., 2020).

Essa técnica consiste na utilização de dois pares de *primers* em uma mesma reação de PCR (reação em cadeia da polimerase), onde os *primers Outer* são não alelo-específicos e são responsáveis por amplificar a região onde se encontra o SNP, enquanto os *primers Inner* são alelo específicos e produzirão fragmentos relacionados a estes (Figura 1) (MEDRANO e OLIVEIRA, 2014).

Figura 1. Representação esquemática de um gel de eletroforese com reação de ARMS-PCR.



Fonte: MEDRANO e OLIVEIRA, 2014

O desenvolvimento dos *primers*, de acordo com as características de cada um pode ser realizado utilizando softwares online, como o Primer 1 (disponível em: [Search Frame \(soton.ac.uk\)](http://SearchFrame.soton.ac.uk)), desenvolvido por Yue e colaboradores (MEDRANO e OLIVEIRA, 2014).

Para que se obtenha bons resultados no uso da técnica, Medrano e Oliveira (2014), descrevem que a estratégia de desenvolvimento e otimização é dividida em três etapas, onde a primeira consiste no uso apenas dos *primers Outer* em reação de PCR, para verificar a formação correta dos *amplicons* previamente determinado para eles e se os mesmos estão amplificando o gene e fragmento alvo a serem analisados; a segunda etapa, consiste no uso de uma reação de RFLP-PCR para identificação do genótipo dos animais, obtenção de amostras controle e comparação e validação dos *primers* para ARMS-PCR desenvolvidos; já a terceira e última etapa, consiste em uma reação contendo os dois pares de *primers* (*Outer* e *Inner*), onde ajustes na reação devem ser realizados até que o genótipo determinado pela ARMS-PCR seja o mesmo das amostras controle por RFLP-PCR (MEDRANO e OLIVEIRA, 2014).

A temperatura de *melting* é considerada como o fator mais importante para atingir a amplificação específica dos alelos, visto que quando trabalhamos com quatro *primers* ao mesmo tempo, há maior chance de formação de bandas inespecíficas na reação (MEDRANO e OLIVEIRA, 2014). A porcentagem de ligações CG nos *primers*, a qualidade do DNA (ácido desoxirribonucleico) e a qualidade dos reagentes utilizados também são fatores que influenciam a reação de ARMS-PCR (MEDRANO e OLIVEIRA, 2014).

Protocolos e set de *primers* da técnica de ARMS-PCR já foram propostos e validados por Rincón e Medrano (2006), para os SNP CAPN1-316_g.5709C>G, CAPN1-4751_g.6545C>T e CAPN1-530_g.4558A>G (RINCÓN e MEDRANO, 2006), e foram utilizados em estudos realizados na Bolívia (PEREIRA et al., 2022), Brasil (CURI et al., 2009; CURI et al., 2010), Colômbia (PEREIRA et al., 2015) e Turquia (KÖK, 2017; KÖK e ATALAY, 2018).

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ABCZ – Associação Brasileira dos Criadores de Zebu. Disponível em: <https://www.abcz.org.br/>. Acesso em: 18/10/2023.

ABIEC - Associação Brasileira das Indústrias Exportadoras de Carne. Disponível em: <https://www.abiec.com.br/>. Acesso em: 18/10/2023.

ABPA – Associação Brasileira de Proteína Animal. Disponível em: <https://abpa-br.org/>. Acesso em: 18/10/2023.

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CAPÍTULO II

Trabalho científico

O trabalho a seguir for redigido de acordo com as normas da revista *Animals*.
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Article

Frequency of Variants Associated with *calpain* and *calpastatin* genes on Brahman Cattle in Brazil

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Simple Summary: This study aimed to evaluate four gene markers (*CAPN1* 316, *CAPN1* 4751, *CAPN3*, and *CAST*) for meat tenderness, an important component of palatability and acceptance of consumers, in Brahman cattle in Brazil, and also to create a protocol with a technique (*ARMS* PCR) considered a simple and low-cost method to determine genotypes with no need for genetic sequencing. Our results showed that the marker *CAST* was the most present in the population studied and may be considered the most selected marker associated with meat tenderness. The *ARMS* PCR protocol developed and described here worked with 100% specificity and was effective in determining the animal's genotype. These results may help producers and breeders to select animals with favorable meat tenderness alleles, using a simple and low-cost method, aiming to obtain products with a better meat characteristic, generating great acceptance by the consumer and profits for production.

Abstract: Meat tenderness is an important component of palatability and certain genetic markers may be used to identify cattle with better meat tenderness, such as those in the *calpain-calpastatin* system. In this study, the allele frequencies of the gene markers *CAPN1* 316, *CAPN1* 4751, *CAPN3*, and *CAST* were assessed in Brazilian Brahman bulls and cows. A total of 108 Brahman DNA samples (54 bulls and 54 cows) were assessed in this study. The samples were submitted to PCR and Sanger direct sequencing to determine the animals' genotype and to compare the sequencing and electropherograms obtained with the previous amplicons of the *ARMS* PCR technique. The gene marker *CAST* was the most prevalent, with 48% (52/108 samples) classified as homozygous AA in this study. For *CAPN1* 316, *CAPN1* 4751, and *CAPN3*, the frequency of favorable alleles was 0% (0/108 samples) for C/C, 0% (0/108 samples) for C/C, and 14% (15/107 samples) for G/G, respectively. Consequently, a protocol and primers for the *ARMS* PCR technique were devised and subsequently validated for the specific *CAST* variant. The *ARMS* PCR technique for the *CAST* gene has proven to be 100% effective, relatively simple, cost-effective, and user-friendly in determining the animal's genotype. These results may help producers and breeders to obtain products with better meat characteristics, generating great acceptance by the consumer and profits for production.

Keywords: *ARMS* PCR, *CAPN1*, *CAPN3*, *CAST*, genetic traits, meat tenderness, prevalence study

1. Introduction

Bos indicus cattle are known to have less desirable growth performance and carcass characteristics [1], and producers and breeders should know that meat quality is a complex subject since many factors such as tender stretch, hormonal growth promotant, marbling, aging, pH, juiciness, and other are involved in the process [2], in which meat tenderness is an important component of palatability [3] and its variation has a significant impact on consumer satisfaction [4]. Since phenotypic data about meat quality before slaughter is restrictive and difficult [3], many gene markers, or single nucleotide polymorphisms (SNP), have been proposed to enhance meat quality, affecting especially tenderness [2,5-6].

The use of SNP has increased genetic improvement as compared to other selection tools [1], panels of SNP, which are available commercially [7] can provide molecular value predictions of breeding [8], and should be stimulated among producers and breeders as an objective to enhance meat quality, consumers satisfaction and to provide more benefits [2].

Certain gene markers may be used to identify cattle with better meat tenderness [2, 5-6], and among them, we can highlight those associated with *calpain* (*CAPN1* 316 g.5709C>T [4], *CAPN1* 4751 g.6545C>T [3], and *CAPN3* c.1538+225G>T [9]) and *calpastatin* (*CAST* c.2832A>G [10]) genes. According to previous studies, the presence of the alleles C (*CAPN1* 316), C (*CAPN1* 4751), G (*CAPN3*), and A (*CAST*) are considered favorable for meat tenderness in Brahman cattle [2-9,11-19].

In Brazil, few studies were performed analyzing the SNP associated with *calpain* and *calpastatin* genes using zebuine, especially Nellore cattle; taurine, and crossbreeds. However, none of them used the Brahman cattle as the object of study [20-25].

Brahman cattle are a zebuine breed characterized by their environment adaptability, maternal ability, longevity, efficient beef production, and docility. Its genetic, composed of Nellore, Guzera, Gir, Krishna Valley, and Indubrasil breeds, is present in more than seventy countries, including Brazil, with good results in crossings with zebuine and taurine breeds for meat and/or dual-purpose systems [26-28].

Some studies about these SNP compared Brahman [2] and Nellore [25] cattle to Angus [2] and Caracu [25] cattle and the results demonstrated that the markers *CAPN1* 316 and *CAPN1* 4751 had a lower percentage in the zebuine breeds and the markers *CAPN3* and *CAST* had a high percentage in the taurine breed and a medium to a high percentage in the zebuine breeds.

The utilization of diverse genotyping techniques, including the MassArray system [3,11], polymerase enzymes [4-6,8-9,19-21,24], direct sequencing [10], RT PCR [15], qPCR [15,23], HRM PCR (high-resolution melting) [17-18], and RFLP PCR (restriction fragment length polymorphism) [14,18,25], has been previously implemented.

The ARMS PCR (amplification refractory mutation system) technique is a relatively simple and cost-effective method to genotype SNP animals without resorting to sequencing procedures [29] and was previously used to detect *CAPN1* 316 and *CAPN1* 4751 gene markers [30].

The objective of this study was to estimate the frequency of favorable alleles for the gene markers *CAPN1* 316, *CAPN1* 4751, *CAPN3*, and *CAST* in important and active reproduction programs of Brazilian Brahman bulls and cows. Furthermore, ARMS PCR primers and protocols were developed and proposed for the *CAPN3* and *CAST* markers, the only two markers in this study for which primers and protocols had not been previously described.

2. Materials and Methods

2.1. Ethical approval statement

All procedures were previously approved by the ethical institutional committee (0194 / 2020 CEUA UNESP) and the samples were collected in a manner that ensures the anonymity of the animals and breeding farms.

2.2. Sample selection

A total of 108 purebred Brahman hair bulb samples, harvested from the tails of 54 bulls and 54 cows, were assessed in this study to evaluate the gene markers *CAPN1* 316, *CAPN1* 4751, *CAPN3*, and *CAST*. All animals were registered in ABCZ [27] and had been utilized in a prior study [31].

2.3. Genomic DNA extraction

The genomic DNA was extracted from hair bulb samples using an in-house method. Initially, 20 hair bulbs from each animal were incubated in 90 μ L of lysis buffer K with proteinase K (0.5 μ L) at 56 °C for 1 hour. Subsequently, 30 μ L of protein precipitation buffer (5.7% ammonium acetate) was added, followed by incubation at 25 °C for 2 min and centrifugation at 1.900 G for 12 min. The resulting supernatant (90 μ L) was mixed with an equal volume of 100% isopropanol and subjected to centrifugation, and the pellet obtained was washed with 70% ethanol. Afterward, the tube was dried out by incubating it at 37 °C for 10 min. The DNA was eluted with 30 μ L of elution buffer (Tris EDTA, pH 8.0) and incubated at 56 °C for 30 min. Subsequently, the DNA samples were stored frozen at -20 °C until PCR procedures.

2.4. PCR, Sanger sequencing, and ARMS-PCR procedures

For the PCR technique, specific primers were designed with the PrimerQuest™ Tool (Integrated DNA Technologies, Inc., Coralville, IA, USA) to amplify the SNP points from *CAPN1* 316, *CAPN1* 4751, *CAPN3*, and *CAST* (Table 1). For *CAPN3* and *CAST* markers, the forward and reverse outer primer of each were used in the PCR technique. The PCR (total volume: 25 μ L) contained 2.5 μ L of template DNA, 0.3 μ M (0.75 μ L) of forward and reverse primers, 12.5 μ L of PCR Master Mix (Promega, CA, USA), and 8.5 μ L of nuclease-free water. A non-template control reaction was used to ensure that PCRs were not contaminated. Thermal cycling conditions were as follows: 95 °C for 10 min; 35 cycles of 95 °C for 45 s, 60.1 °C for 60 s, 72 °C for 45 s; and 72 °C for 10 min.

Amplicons were analyzed via 1.5% agarose gel electrophoresis and the products with the correct size were purified using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich®, St. Louis, MA, USA), according to the manufacturer's instructions. To sequence the DNA, 10 μ L of each purified PCR product, 5 μ L of the forward primers (Table 1), and the BigDye® Terminator Cycle Sequencing Kit (Life Technologies™, Carlsbad, CA, USA) were used. The data generated from sequencing were analyzed in the Geneious® software (Biomatters® Ltd, Auckland, New Zealand) and were compared with the *CAPN1* 316 (AH009246.3), *CAPN1* 4751 (AH009246.3), *CAPN3* (NC037337.1), or *CAST* (NC037334.1) sequences deposited in GenBank™ (<https://www.ncbi.nlm.nih.gov/genbank/>).

Table 1. PCR and sequencing primer sets for the *CAPN1* 316, *CAPN1* 4751, *CAPN3*, and *CAST* markers, and *ARMS* PCR primers designed for *CAPN3* and *CAST* gene markers used in this study.

Primer sets	Primer sequences (5' – 3')	Product	GenBank ^T M
<i>CAPN1</i> 316	GTGTGAACTGGGTCTGGGACTGCCCT TGTTCCAGTTGCGGAACCTCTGGCTC TT	543 bp ¹	AH009246. 3
<i>CAPN1</i> 4751	AAGACCTGCGGACCACGGGCTTCAG CCT TCGCGCTGGGCGGCCGTTAAGAAGA AAA	483 bp	AH009246. 3
<i>CAPN3</i> (<i>outer</i>) ²	CTAGCATGCCCTTTGGCAAGTTTCCA TC TGGAGCTCCCTACCCCATATCCTCTT CC	590 bp	NC0373377 .1
<i>F inner</i> (G)	GTGTGAGTGTGAGCAAGCATGTGGG C	349 bp	
<i>R inner</i> (T)	CCTTGCCCAACCAGAGACCATACGCT GAAACTGAATTTGTCTGGTTCACCTG TGTT	293 bp	
<i>CAST</i> (<i>outer</i>) ²	GCGTTTGTCTCTTCTGACTTGAGTCAA G	537 bp	NC037334. 1
<i>F inner</i> (A)	CTGATTTCTCATGACCCCTTTCCTCGT A	263 bp	
<i>R inner</i> (G)	AAACATCAAACACAGTCCACAAGGC C	328 bp	

¹Base pairs

²Primer used for PCR and Sanger direct sequencing

The *ARMS* PCR with a final volume of 30 µL contained: 3 µL of template DNA, 0.11 µM (0.33 µL) of each forward and reverse outer primer, 0.08 µM (0.24 µL) of each forward and reverse inner primer (Table 1), 15 µL of GoTaq Green PCR Master Mix (Promega, Madison, WI, USA), and 10.86 µL of nuclease-free water. The reaction with a template was also performed to check for possible contaminations in the PCR mix. The thermocycling condition was similar to that described for the PCR. Amplicons were analyzed via 4% agarose gel electrophoresis and the genotype, according to their preview fragments. In the end, the genotypes of all animals identified by *ARMS* PCR were compared with those obtained through Sanger sequencing described above.

2.5. Limitations of the Study

The main limitations of the study were the impossibility of carrying out meat tenderness analyses after the slaughter of the animals evaluated in the current studies, as the selected animals are alive and participate in active reproduction programs and the collection of muscle samples was not authorized by the owners. Moreover, the study's limitation extends to the sample size of animals assessed, potentially compromising its representation of the Brazilian Brahman herd. Nevertheless, it is noteworthy that these animals play a pivotal role in breeding

programs, serving as crucial contributors by functioning as semen or oocyte donors in artificial insemination and *in vitro* fertilization processes.

3. Results

According to the Sanger direct sequencing results and electropherograms analysis, for *CAPN1* 316, 97% (105/108) and 3% (3/108) of the samples were classified as GG and CG genotypes, respectively; for *CAPN1* 4751, 94% (102/108) were TT, and 6% (6/108) were CT; for *CAPN3*, 14% (15/107) were GG, 49% (52/107) were GT, and 37% (40/107) were TT; and for *CAST*, 48% (52/108) were AA, 32% (35/108) were AG, and 20% (21/108) were GG (Table 2).

Table 2. Genotypes observed in 108 Brahman DNA samples for the *CAPN1* 316, *CAPN1* 4751, *CAPN3*, and *CAST* gene markers.

<i>CAPN1</i> 316 g.5709C>G	N (%)	<i>CAPN3</i> c.1538+225G>T	N (%)
GG	105 (97%)	GG	15 (14%)
CG	3 (3%)	GT	52 (49%)
CC	ND ¹	TT	40 (37%)
Total:	108 (100%)	Total:	107 (100%)

<i>CAPN1</i> 4751 g.6545C>T	N (%)	<i>CAST</i> c.2832A>G	N (%)
TT	102 (94%)	AA	52 (48%)
CT	6 (6%)	AG	35 (32%)
CC	ND	GG	21 (20%)
Total:	108 (100%)	Total:	108 (100%)

¹ ND, not detected.

When we compare the results of the markers *CAPN1* 316, *CAPN1* 4751, and *CAPN3* with the favorable allele AA of the *CAST* marker, 48% (51/105) of GG samples and 1% (1/3) of CG samples for *CAPN1* 316 were classified as AA for *CAST*, respectively; for *CAPN1* 4751, 50% (51/108) of TT and 17% (1/6) of CT were AA for *CAST*; and for *CAPN3*, 47% (7/15) of GG, 44% (23/52) of GT, and 55% (22/40) of TT were AA for *CAST*.

Considering that the AA allele of the *CAST* marker has previously been linked to meat tenderness in Brahman cattle [5-6,8-9,15,17], given the heightened prevalence of the desired genotype (AA) for the *CAST* SNP within the studied population in our current investigation, and according to the objectives of the study, we developed and validated an ARMS PCR assay specifically designed for the identified *CAST* variant.

The ARMS PCR developed for the *CAST* SNP performed effectively, successfully detecting all three possible genotypes, in all of our samples (108 samples), through the analysis of the obtained amplicons based on their respective fragment sizes, i.e., AG animals formed a 537 bp for outer primers, 328 bp for inner (G), and 263 bp for inner (A); GG animals a 537 bp for outer primer and 328 bp for inner (G); and AA animals a 537 bp for outer primer and 263 bp for inner (A) (Figure 1). The validation of ARMS PCR was accomplished through Sanger sequencing (validation step), demonstrating the concordance of genotypes between both methodologies (Figure 2).

The primers developed and described here for the *CAPN3* marker did not work detecting and classifying all three possible genotypes through the analysis of the obtained amplicons based on their respective fragment sizes, which means that the primers were unspecific.

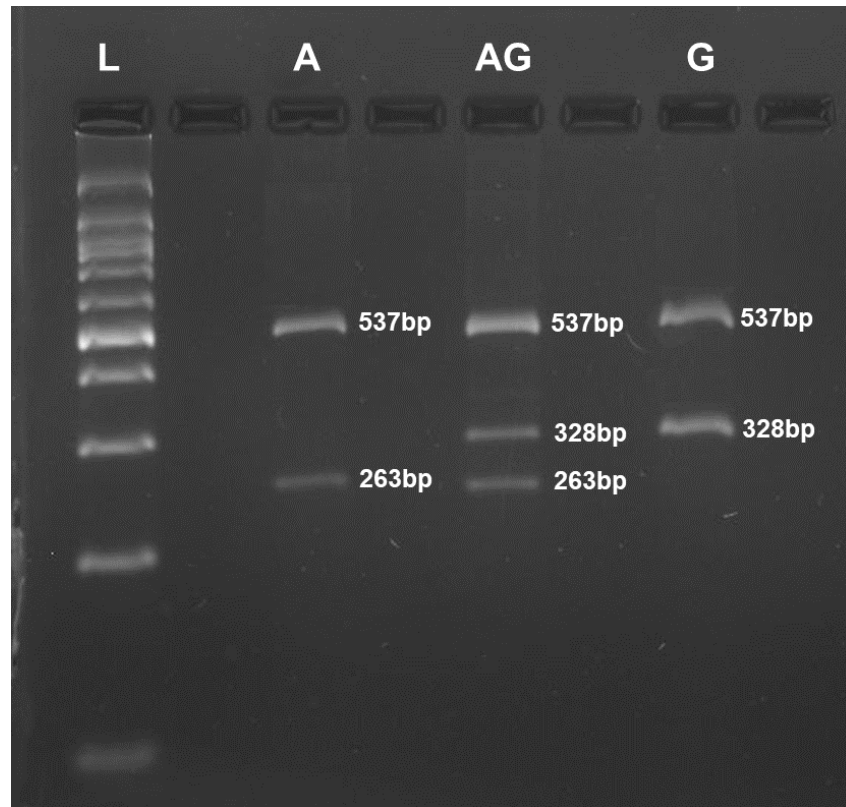


Figure 1. Agarose gel electrophoresis (4%) showing *ARMS* PCR targeting the *CAST* c.2832A>G variant. Note, (L) Low ladder of 100 bp; (A) homozygous Brahman for allele A (two bands of 537 and 263 bp); (AG) heterozygous Brahman (three bands of 537, 328 and 263 bp); (G) homozygous Brahman for allele G (two bands of 537 and 328 bp).

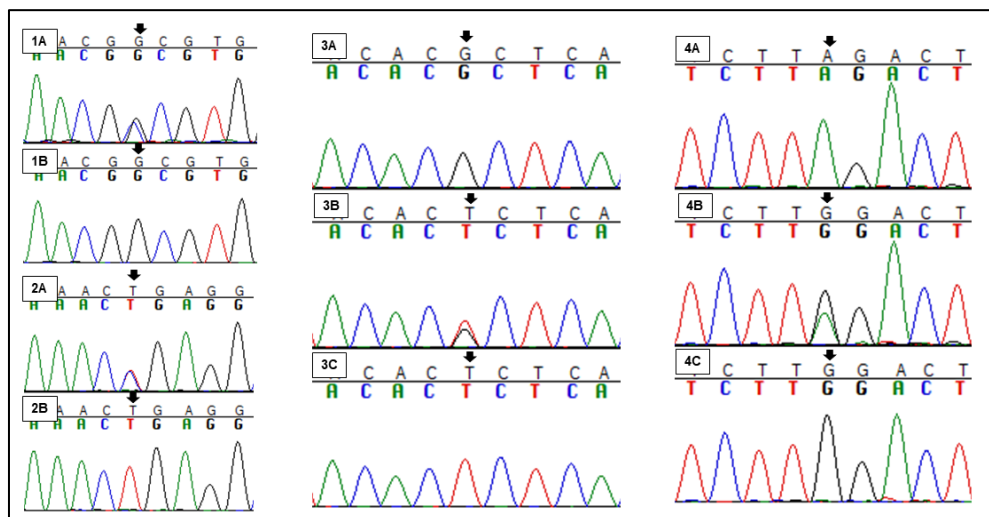


Figure 2. Partial electropherograms from (1) *CAPN1* 316: (1A) animal allele C/G (heterozygous) and (1B) animal allele G/G; (2) *CAPN1* 4751: (2A) animal allele C/T (heterozygous) and (2B) animal allele T/T; (3) *CAPN3*: (3A) animal allele G/G, (3B) animal allele G/T (heterozygous), and (3C) animal allele T/T; and (4) *CAST*: (4A) animal allele A/A, (4B) animal allele A/G (heterozygous), and (4C) animal allele G/G. **Black arrow:** point of the SNP.

4. Discussion

Page and collaborators (2002) [4], described the SNP *CAPN1* 316, where the presence of the allele CC is considered favorable for meat tenderness [3-7,21]. The prevalence observed in this study (97% [105/108 samples] of the samples were classified as GG allele and 3% [3/108 samples] as CG allele) (Table 2) is similar to those studies performed in Argentina [13], Australia [2,5-6,8,15], Bolivia [19], Colombia [17-18], the USA [3,11], and Puerto Rico [16]. The similarity of the results may be associated with the fact that Brazilian Brahman cattle genetics is composed of Australian, American, and African lineages [27-28,32] and that Brahman is the second beef breed that most exports semen to Latin America and Asia in Brazil [32]. A low percentage of CC animals was also observed by Curi and collaborators (2010) and Pinto and collaborators (2010), who used Nellore cattle, a zebuine breed that composes Brahman genetics [27-28,32] in their study (0% and 0%, respectively) [21-22], reinforcing that GG allele is fixed in the Brahman breed and zebuine cattle [3] and that this SNP is associated with taurine breeds and crossbreeds [21].

The SNP *CAPN1* 4751 was described by White and colleagues (2005), where the presence of the allele CC is also favorable for meat tenderness [3,5-7], and it is the only marker described to influence the shear force in case of heterozygosis (allele CT) [3]. About 94% of the Brazilian Brahman were classified as TT allele and 6% as CT allele, these findings were different from the other studies performed in Australia [2,5-6,8,15], Bolivia [19], Colombia [17-18], the USA [7], since CC allele animals were found with a prevalence varying from 1% [7] to 28.9% [17]; and similar to those in Argentina [13], Puerto Rico [16], and the USA [3,12], which no CC allele animals were observed. This may infer that some Brazilian animals are descendants from lineages that this SNP was not used as a trait marker selector, which can explain no allele CC animals in this population studied, and reinforces that segregation of this SNP is observed in Brahman cattle [11]. This low percentage of the allele CC was also observed in the Nellore breed in studies performed in Brazil, varying from 0% [20,25] to 3.2% [22].

Barendse and colleagues (2008) described the SNP *CAPN3* and considered it the most prevalent in zebuine and crossbreeds than in taurine breeds [9]. According to studies, the presence of the allele GG is favorable for meat tenderness [5-6,9], however, there are some controversies about the roleplay of *CAPN3* in meat tenderness post-mortem [5-6]. Studies about this SNP were only performed with Australian Brahman cattle [2,5-6,8-9,14-15], where the prevalence of favorable alleles varied from 27,03% [9] to 37,68% [2,5,8,15], which is different from what was observed in this study (14% were classified as GG allele, 49% were GT, and 37% were TT), where a lower frequency of allele GG was found. Since the Brazilian Brahman genetics is composed of Australian, American, and African lineages [27-28,32], and the studies about *CAPN3* were performed in Australian Brahman cattle [2,5-6,8-9,14-15], we can assume that this SNP is not frequently used as a trait mark selector in Brazilian animals, even with a higher prevalence in zebuine cattle [9] and influence of Australian lineages. No study about *CAPN3* was performed in Brazil, so, this study is the first one to evaluate the prevalence of this SNP in Brazilian cattle population.

Lastly, the marker *CAST* was the most prevalent in this study to present the favorable allele AA [5-6]. According to the sequencing results and electropherograms, 48% (52/108) of the samples were classified as AA allele, 32% (35/108) were AG, and 20% (21/108) were GG. The prevalence of the AA allele in this study was higher than those observed in Australia [2,5-6,8-9,15] and Colombia [17], where the prevalence was from 30,38% [9] to 44,21% [2,5-6,8,15]; similar and lower to other studies in Australia [14] and Colombia [18], with a prevalence of 48,14% and 52%, respectively. With this in mind, we can presume that this SNP may be the most selected marker associated with meat tenderness in Brazilian Brahman lineages and animals, and most of the lineages may have an Australian influence, since the highest prevalence (48,14%) was observed in a study with Australian Brahman cattle [14]. Studies in Brazil with the Nellore breed observed a prevalence of 30,77% [25] to 34,2% [20] of the favorable

allele AA [20,23,25]. The results of the SNP *CAST* in this study, in other studies worldwide, and Brazil reinforce that this marker can be used as a trait selector for Brahman, Nellore, and crossbreeds for meat tenderness with a medium to high prevalence of favorable allele [2,5-6,8-9,14-15,17-18,20,23,25].

The four SNP (*CAPN1* 316, *CAPN1* 4751, *CAPN3*, and *CAST*) analyzed in this study were described and proved to enhance meat tenderness by many studies worldwide using not only the Brahman breed but also zebuine, taurine, crossbreeds, and breeds that compound it's genetic, i.e., the Nellore breed [2-9, 11-25]. Robinson and colleagues' study described the effect of these SNP over other meat qualities, such as marbling, juiciness, and acceptance by the consumer [2]. Due to that, we can speculate that maybe not only meat tenderness could be enhanced using the data shown in this study, but also other meat qualities that influence consumer acceptance.

The ARMS PCR technique is regarded as a simple and cost-effective method for genotyping SNP without the necessity for advanced and expensive techniques, such as genetic sequencing [29], and has been proposed by Rincón and Medrano for the gene markers *CAPN1* 316 and *CAPN1* 4751 [30].

As part of the objective of this study, the authors proposed to develop and validate primers and protocols for *CAPN3* and *CAST*, the only two markers that do not have a description of ARMS PCR primers and protocols. Our *CAPN3* primers were unspecific, which means that were incapable of detecting and classifying all three possible genotypes for this variant. According to Medrano and Oliveira, when working with all four primers in the same reaction, the chance of unspecific amplifications is bigger, and the melting temperature is considered the most important factor in achieving an allele-specific amplification [29]. The authors followed the recommendations from Medrano and Oliveira's article to achieve success in the ARMS PCR technique, changing the melting temperature and performing a touchdown PCR, but the results were the same. Other factors, such as DNA quality, reagents equilibrium, and primers balancing (percentage of CG) can also influence the results, and we can not ensure what could influenced our reaction, since all measures were taken to avoid this result.

For *CAST*, the primers and protocol developed worked with 100% efficiency in the classification and determination of the animals' genotypes, forming amplicons according to their compared sequencing results and preview fragments. This is the first study to describe primers and protocols for the SNP *CAST* using the ARMS PCR technique.

5. Conclusions

In conclusion, the SNP *CAST* c.2832A>G was the most prevalent marker to present the favorable allele AA in the Brazilian Brahman cattle population examined in this study. The ARMS PCR technique developed for genotyping the *CAST* marker has proven to be straightforward, specific, and effective in determining the animals' genotype. According to our results, it might be employed to screen for the more prevalent marker observed in the population assessed in the present study and previously associated with tenderness by other authors. The association of these results and knowledge of the animal's genotype involved in the breeding may help producers and breeders, aiming to obtain products with better meat characteristics, thus generating great acceptance by the consumer and profits for production.

Author Contributions: LGA and JPOF contributed to the conception and design of the study; JPOF contributed to the sample collection; LGA, GRL, and LMSS contributed to the processing of the samples; LGA, ASB, and JPOF contributed to writing the first drafts of the manuscript; JPOF contributed to directing the project. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committee of São Paulo State University (UNESP) (protocol code 0194/2020 on 12 December 2020).

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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- *New nucleic acid sequences* must be deposited into an acceptable repository such as [GenBank](http://www.ncbi.nlm.nih.gov/genbank), [EMBL](http://www.ebi.ac.uk/EMBL), or [DDBJ](http://www.ddbj.nig.ac.jp). Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, ...) must be deposited either in the [GEO database](http://www.ncbi.nlm.nih.gov/geo) or in the NCBI's [Sequence Read Archive \(SRA\)](http://www.ncbi.nlm.nih.gov/sra).
- *New microarray data* must be deposited either in the [GEO](http://www.ncbi.nlm.nih.gov/geo) or the [ArrayExpress](http://www.ncbi.nlm.nih.gov/arrayexpress) databases. The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool [SPIN](http://www.uniprot.org/spin)). Annotated protein structure and its reference sequence must be submitted to [RCSB of Protein Data Bank](http://www.rcsb.org).

All sequence names and the accession numbers provided by the databases must be provided in the Materials and Methods section of the article.

Deposition of Proteomics Data

Methods used to generate the proteomics data should be described in detail and we encourage authors to adhere to the "[Minimum Information About a Proteomics Experiment](#)". All generated mass spectrometry raw data must be deposited in the appropriate public database such as [ProteomeXchange](#), [PRIDE](#) or [jPOST](#). At the time of submission, please include all relevant information in the materials and methods section, such as repository where the data was submitted and link, data set identifier, username and password needed to access the data.

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Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

Institutional Review Board Statement

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the [Declaration of Helsinki of 1975](#), which was revised in 2013. According to point 23 of this declaration, approval from the local Institutional Review Board (IRB) or another appropriate ethics committee must be obtained before undertaking the research to confirm that the study meets national and international guidelines. As a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board must be stated in the 'Institutional Review Board Statement' Section of the article.

Example of an Institutional review board statement: "The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code) on [date of approval]."

For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed whether their anonymity is assured, why the research is being conducted, how their data will be used, and if there are any risks involved in participating. As with all research involving humans, ethical approval from an appropriate ethics committee must be obtained prior to conducting the study. If ethical approval is not required, authors must either provide an exemption from the ethics committee or cite the local or national legislation that indicates ethics approval is not required for this type of study. When a study has been granted exemption, the name of the ethics committee that provided this should be stated in the 'Institutional Review Board Statement' Section with a full explanation for the rejection of ethical approval.

Informed Consent Statement

Manuscripts reporting studies involving human participants, human data, or human tissue must include a **statement of informed consent for participation** in

research. Verbal informed consent to participate in a study can be acceptable under some circumstances (such as in ethnographic studies). The authors must explain the rationale for using this kind of consent in the “Informed Consent Statement” Section. For verbal informed consent, a copy of the script used must be provided during the submission stage.

For all manuscripts that include identifying patient/participant information (personal details, images, or videos relating to an individual person), written **informed consent for the publication** of these details must be obtained from patients/participants (or their relatives/guardians) before submitting to an MDPI journal. A blank version of the form used to obtain permission (without the patient/participant names or signature) should be provided upon submission. You may refer to our [template permission form](#) and provide an appropriate form after consulting with your affiliated institution.

For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under open access license. To respect patients’/participants’ and any other individuals’ privacy, please do not send signed forms.

Private information identifying participants need not be included unless the identifiable materials are of relevance to the research (e.g., photographs of participants’ faces that show a particular symptom). Patients’/participants’ initials or other personal identifiers must not appear in any images. Patient/participant details must be anonymized as much as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. Steps necessary to protect privacy may include de-identifying data, adding noise, or blocking portions of the database. Editors reserve the right to reject any submission that does not meet these requirements.

The Editorial Office reserves the right to request further documentation when necessary. The submitted manuscript will be scrutinized by the Editorial Office, and upon request, documentary evidence (signed consent forms and any related discussion documents from the ethics board) must be provided.

Example of an Informed Consent Statement: “Informed consent for participation was obtained from all subjects involved in the study.” OR “Informed consent for participation is not required as per local legislation [provide local legislation].” OR “Verbal informed consent was obtained from the participants. Verbal consent was obtained rather than written because [state the reason]”, OR “Informed consent for publication was obtained from all identifiable human participants.”

Requirements for Studies on Vulnerable Groups and Organ Transplants

If a study involves vulnerable groups, the manuscript will undergo an additional review by the editorial office. If requested, the author must provide documentary evidence, including blank consent forms and any related discussion documents from the ethics board or other relevant bodies. Additionally, when studies describe groups by race, ethnicity, gender, disability, disease, etc., an explanation regarding why such categorization was needed must be clearly stated in the article.

Articles describing human organ transplantation studies are subject to all policies for research involving human subjects. Additionally, the authors must specify the institution(s), clinic(s), or department(s) from which the organs or tissues were sourced. MDPI does not accept manuscripts that report data on organs and/or other materials obtained from illegal commercial activity, executed prisoners, or other unethical practices relating to organ donations. Manuscripts addressing this practice, such as editorials or reports on its secondary consequences, may be considered at the discretion of the Editor-in-Chief but require a written appeal to the editorial office before submission. For further resources on organ transplantation, MDPI follows the glossary maintained by the Organ Procurement and Transplantation Network (<https://optn.transplant.hrsa.gov/patients/glossary/>).

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs [1]':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Authors must include details on housing, husbandry and pain management in their manuscript.

MDPI endorses the ARRIVE guidelines (arriveguidelines.org/) for reporting experiments using live animals. Authors and reviewers must use the ARRIVE guidelines as a checklist, which can be found at <https://arriveguidelines.org/sites/arrive/files/documents/Author%20Checklist%20-%20Full.pdf>. The journal *Nutrients* requires authors to submit the completed checklist at submission, and it will be made available to reviewers. Editors reserve the right to reject submissions that do not adhere to these guidelines based on ethical or animal welfare concerns, or if the procedure described does not appear to be justified by the value of the work presented.

For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [2], American Association for Laboratory Animal Science [3] or European Animal Research Association [4].

If national legislation requires it, studies involving vertebrates or higher invertebrates must only be carried out after obtaining approval from the appropriate ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be stated in Section 'Institutional Review Board Statement'. Research procedures must be carried out in accordance with national and institutional regulations. Statements on animal welfare should confirm that the study complied with all relevant legislation. Clinical studies involving animals and interventions outside of

routine care require ethics committee oversight as per the American Veterinary Medical Association. If the study involved client-owned animals, informed client consent must be obtained and certified in the manuscript report of the research. Owners must be fully informed if there are any risks associated with the procedures and that the research will be published. If available, a high standard of veterinary care must be provided. Authors are responsible for correctness of the statements provided in the manuscript.

If ethical approval is not required by national laws, authors must provide an exemption from the ethics committee, if one is available. Where a study has been granted exemption, the name of the ethics committee that provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation on why the ethical approval was not required.

If no animal ethics committee is available to review applications, authors should be aware that the ethics of their research will be evaluated by reviewers and editors. Authors should provide a statement justifying the work from an ethical perspective, using the same utilitarian framework that is used by ethics committees. Authors may be asked to provide this even if they have received ethical approval.

1. NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: <https://www.animaethics.org.au/three-rs>
2. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf
3. American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: <https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use>
4. European Animal Research Association. EU regulations on animal research. Available online: <https://www.eara.eu/animal-research-law>

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The MLH1⁺ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the [Convention on Biological Diversity](#) and the [Convention on the Trade in Endangered Species of Wild Fauna and Flora](#).

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana*, *Nicotiana benthamiana*, *Oryza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopsis mutant lines (SALKxxxx, SAILxxxx,...) were kindly provided by Dr. XXX, institute, city, country).

Clinical Trials Registration

Registration

MDPI follows the International Committee of Medical Journal Editors (ICMJE) [guidelines](#) which require and recommend registration of clinical trials in a public trials registry at or before the time of first patient enrollment as a condition of consideration for publication.

Purely observational studies do not require registration. A clinical trial not only refers to studies that take place in a hospital or involve pharmaceuticals, but also refer to all studies which involve participant randomization and group classification in the context of the intervention under assessment.

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register and cite a reference to the registration in the Methods section. Suitable databases include [clinicaltrials.gov](#), [the EU Clinical Trials Register](#) and those listed by the World Health Organisation [International Clinical Trials Registry Platform](#).

Approval to conduct a study from an independent local, regional, or national review body is not equivalent to prospective clinical trial registration. MDPI

reserves the right to decline any paper without trial registration for further peer review. However, if the study protocol has been published before the enrolment, the registration can be waived with correct citation of the published protocol.

CONSORT Statement

MDPI requires a completed CONSORT 2010 [checklist](#) and [flow diagram](#) as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (<http://www.consort-statement.org>) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist.

Dual Use Research of Concern

MDPI follows the practical framework defined in [Guidance for Editors: Research, Audit and Service Evaluations](#) and introduced by the Committee on Publication Ethics (COPE). Research that could pose a significant threat, with broad potential consequences to public health or national security, should be clearly indicated in the manuscript, and potential dual-use research of concern should be explained in the cover letter upon submission. Potential areas of concern include but are not limited to biosecurity, nuclear and chemical threats, and research with a military purpose or application, etc. For these manuscripts to be considered for peer review, the benefits to the general public or public health must outweigh the risks. The authors have a responsibility to comply with relevant national and international laws.

Sex and Gender in Research

We encourage our authors to follow the [‘Sex and Gender Equity in Research – SAGER – guidelines’](#) and to include sex and gender considerations where relevant. Authors should use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Article titles and/or abstracts should indicate clearly what sex(es) the study applies to. Authors should also describe in the background, whether sex and/or gender differences may be expected; report how sex and/or gender were accounted for in the design of the study; provide disaggregated data by sex and/or gender, where appropriate; and discuss respective results. If a sex and/or gender analysis was not conducted, the rationale should be given in the Discussion. We suggest that our authors consult the full [guidelines](#) before submission.

Borders and Territories

Potential disputes over borders and territories may have particular relevance for authors in describing their research or in an author or editor correspondence address, and should be respected. Content decisions are an editorial matter and where there is a potential or perceived dispute or complaint, the editorial team will attempt to find a resolution that satisfies parties involved.

MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Publication Ethics Statement

Animals is a member of the Committee on Publication Ethics ([COPE](#)). We fully adhere to its [Code of Conduct](#) and to its [Best Practice Guidelines](#).

The editors of this journal enforce a rigorous peer review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Animals* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Animals* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- The journal accepts exact translations of previously published work. All submissions of translations must conform with our [policies on translations](#).
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our [policy regarding Updating Published Papers](#).
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the [Rights and Permissions](#) page.
- Plagiarism, data fabrication and image manipulation are not tolerated.

- **Plagiarism is not acceptable** in *Animals* submissions.

Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

All MDPI submissions are checked for plagiarism using the industry standard software iThenticate. If plagiarism is detected during the

peer review process, the manuscript may be rejected. If plagiarism is detected after publication, an investigation will take place and action taken in accordance with our policies.

- **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

Citation Policy

Authors should ensure that where material is taken from other sources (including their own published writing) the source is clearly cited and that where appropriate permission is obtained.

Authors should not engage in excessive self-citation of their own work.

Authors should not copy references from other publications if they have not read the cited work.

Authors should not preferentially cite their own or their friends', peers', or institution's publications.

Authors should not cite advertisements or advertorial material.

In accordance with COPE guidelines, we expect that "original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations." This condition also applies to an author's own work. COPE have produced a discussion document on [citation manipulation](#) with recommendations for best practice.

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Reviewer Suggestions

During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily

approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last three years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper. For detailed information regarding the qualifications and responsibilities of the reviewers, please visit <https://www.mdpi.com/reviewers>.

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Extensive English Editing

It is the authors' responsibility to submit their work in correct English. The APC includes only minor English editing, conducted by native English speakers. The APC does not include extensive English editing. If extensive editing is required, your paper could be returned to you at the English editing stage of the publication process. This could delay the publication of your work. You may have your work reviewed by an experienced English-speaking colleague or use a paid language-editing service before submitting your paper for publication. We offer rapid English editing, completed in 1 day, here: [Author Services](#).

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Preprints and Conference Papers

Animals accepts submissions that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates *Preprints*, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* [instructions for authors](#) for further information.

Expanded and high-quality conference papers can be considered as articles if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *Animals* does not publish pilot studies or studies with inadequate statistical power.

Unpublished conference papers that do not meet the above conditions are recommended to be submitted to the [Proceedings Series journals](#).

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Authorship

MDPI follows the International Committee of Medical Journal Editors ([ICMJE](#)) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or reviewing it critically for important intellectual content; AND
- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. More detailed guidance on authorship is given by the [International Committee of Medical Journal Editors \(ICMJE\)](#).

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

For more details about authorship please check [MDPI ethics website](#).

Editorial Independence

Lack of Interference with Editorial Decisions

Editorial independence is of utmost importance and MDPI does not interfere with editorial decisions. All articles published by MDPI are peer reviewed and assessed by our independent editorial boards, and MDPI staff are not involved in decisions to accept manuscripts. When making an editorial decision, we expect the academic editor to make their decision based only upon:

- The suitability of selected reviewers;
- Adequacy of reviewer comments and author response;
- Overall scientific quality of the paper.

In all of our journals, in every aspect of operation, MDPI policies are informed by the mission to make science and research findings open and accessible as widely and rapidly as possible.

Editors and Editorial Staff as Authors

Editorial staff or editors shall not be involved in processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers. Decisions will be made by other Editorial Board Members who do not have a conflict of interest with the author. Journal staff

are not involved in the processing of their own work submitted to any MDPI journals.

Conflicts of Interest

According to The International Committee of Medical Journal Editors, “Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors’ access to all of the study’s data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose.”

All authors must disclose all relationships or interests that could inappropriately influence or bias their work. Examples of potential conflicts of interest include but are not limited to financial interests (such as membership, employment, consultancies, stocks/shares ownership, honoraria, grants or other funding, paid expert testimonies and patent-licensing arrangements) and non-financial interests (such as personal or professional relationships, affiliations, personal beliefs).

Authors can disclose potential conflicts of interest via the online submission system during the submission process. Declarations regarding conflicts of interest can also be collected via the [MDPI disclosure form](#). The corresponding author must include a summary statement in the manuscript in a separate section “Conflicts of Interest” placed just before the reference list. The statement should reflect all the collected potential conflicts of interest disclosures in the form.

See below for examples of disclosures:

Conflicts of Interest: Author A has received research grants from Company A. Author B has received a speaker honorarium from Company X and owns stocks in Company Y. Author C has been involved as a consultant and expert witness in Company Z. Author D is the inventor of patent X.

If no conflicts exist, the authors should state:

Conflicts of Interest: The authors declare no conflicts of interest.

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Editorial Procedures and Peer Review

Pre-check

Immediately after submission, the journal’s Managing Editor will perform the technical pre-check to assess:

- Overall suitability of the manuscript to the journal/section/Special Issue;
- Manuscript adherence to high-quality research and ethical standards;
- Standards of rigor to qualify for further review.

The academic editor (i.e., the Editor-in-Chief in the case of regular submissions, the Guest Editor in the case of Special Issue submissions, or an Editorial Board member in the case of a conflict of interest and of regular submissions if the

Editor-in-Chief allows) will be notified of the submission and invited to perform an editorial pre-check. During the editorial pre-check phase, the academic editor will assess the suitability of the submission with respect to the scope of the journal, as well as the overall scientific soundness of the manuscript, including the relevance of the references and the correctness of the applied methodology. Academic editors can decide to reject the manuscript, request revisions before peer review, or continue with the peer review process and recommend suitable reviewers.

Peer Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board Members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past three years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript. For more details about potential conflicts of interest, please check here, [https://www.mdpi.com/reviewers# bookmark9](https://www.mdpi.com/reviewers#bookmark9).

Optional Open Peer Review

The journal operates optional open peer review: *Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports.* Authors can alter their choice for open peer review at any time before publication, but once the paper has been published changes will only be made at the discretion of the *Publisher* and *Editor-in-Chief*. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their research. To guarantee impartial refereeing, the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- *Accept after Minor Revisions:*
The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.
- *Reconsider after Major Revisions:*
The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. A maximum of two rounds of major revision per manuscript is normally provided. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be

returned to the reviewer for further comments. If the required revision time is estimated to be longer than 2 months, we will recommend that authors withdraw their manuscript before resubmitting so as to avoid unnecessary time pressure and to ensure that all manuscripts are sufficiently revised. (Please detail the revisions that have been made, citing line number and exact change, so that the editor can check the changes expeditiously. Simple statements like 'done' or 'revised as requested' will not be accepted, unless the change is simply a typographical error).

- *Reject and Encourage Resubmission:*
If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.
- *Reject:*
The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments using an [appeal form](#). Appeals can only be submitted following a "reject and decline resubmission" decision and should be submitted within three months from the decision date. Failure to meet these criteria will result in the appeal not being considered further. The *Managing Editor* will forward the manuscript and related information (including the identities of the referees) to a designated *Editorial Board Member*. The Academic Editor being consulted will be asked to provide an advisory recommendation on the manuscript and may recommend acceptance, further peer review, or uphold the original rejection decision. This decision will then be validated by the *Editor-in-Chief*. A reject decision at this stage is final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.com website.

Please read detailed Editorial Process [here](#).

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Transfer Service

A manuscript transfer provides you with a convenient method of resubmitting your manuscript file and any reviewer comments to another journal within our publishing portfolio.

We are committed to helping authors find the right home for their research, and we will provide authors with guidance and technical support through all stages of

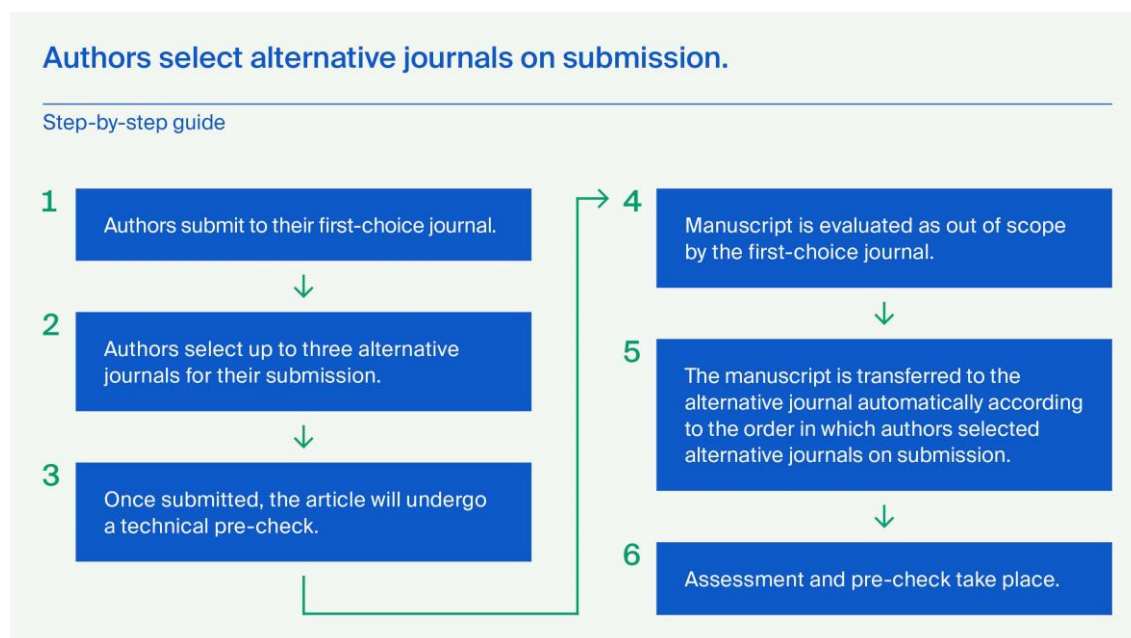
the transfer process. Authors will be able to choose to transfer in the following two situations:

1. Select alternative journals on submission.

We now ask all authors to select up to three alternate journals during the submission process. The suggested alternative journals will be ordered according to the authors' preference. If a paper is evaluated as out of scope by the first-choice journal, it will be transferred to the first alternative journal automatically. If the first alternative journal also rejects the paper because it is out of scope, it will be transferred to the second alternative journal automatically, and so on.

The information below applies for the transfer of manuscripts when your paper is rejected and automatically transferred to your selected alternative journal.

Note: If you did not select an alternative journal during submission, the below does not apply.



How do I select an alternative journal?

Authors can use our [Journal Finder](#) tool to identify suitable journals in our portfolio. We suggest you select alternative journals based on the scope and subject area of your manuscript.

All of our journals are listed [here](#), and you can find out more about each journal by clicking through to the journal homepage.

Do I have to pay to transfer my manuscript?

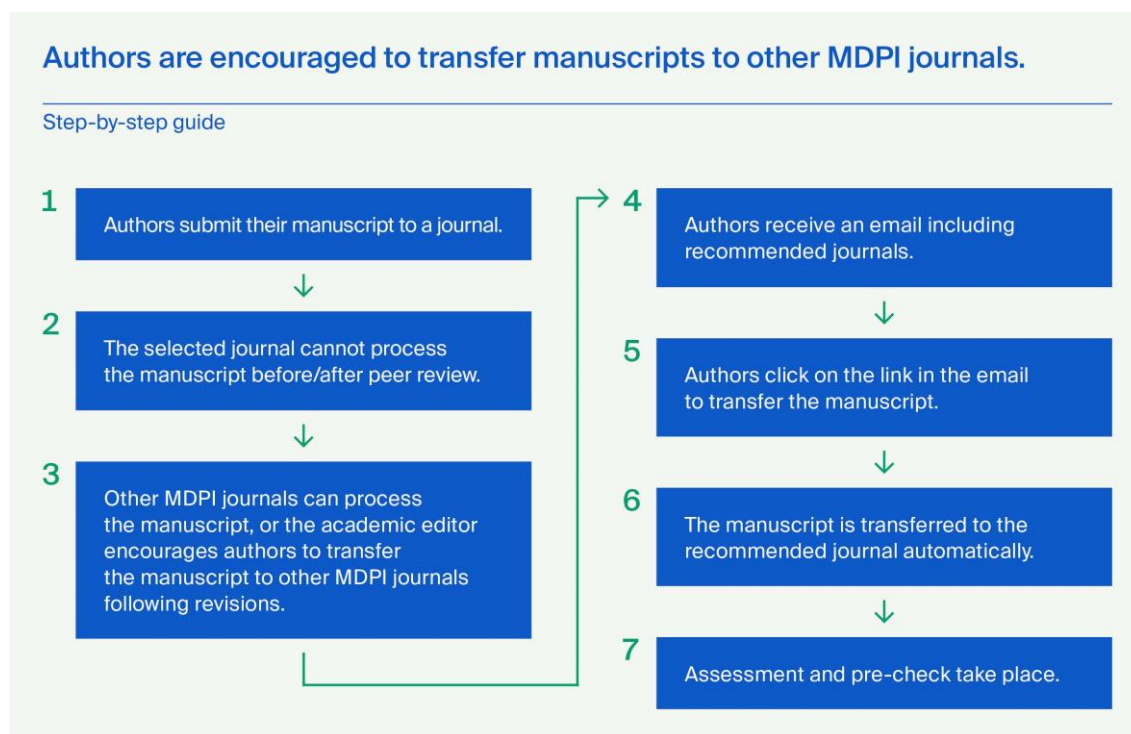
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CAPÍTULO III

Considerações finais

Em conclusão, o SNP *CAST_c.2832A>G* foi o marcador mais prevalente marcador a apresentar o alelo favorável A/A na população brasileira de Brahman analisada neste estudo. A técnica de *ARMS-PCR* desenvolvida para o biomarcador *CAST* provou ser específica e efetiva em determinar o genótipo do animal. A associação destes resultados e o conhecimento dos genótipos dos animais envolvidos no cruzamento, podem ajudar produtores e criadores, visando a obtenção de produtos com melhores características de carne, além de gerar melhor aceitação pelo consumidor e lucros para produção.

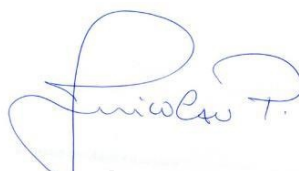
ANEXOS

ATESTADO

Atesto que o Projeto "Screening de mutações associadas aos genes calpain e calpastatin na produção em bovinos da raça Brahman" **Protocolo CEUA 0194/2020**, a ser conduzido por Lukas Garrido Albertino, responsável/orientador José Paes de Oliveira-Filho, para fins de pesquisa científica/ensino - encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal - CONCEA.

Finalidade	PESQUISA CIENTÍFICA
Vigência do projeto	01/03/2021 a 29/02/2024
Nome Comum / Espécie / Linhagem	BOVINA / BOS INDICUS / Zebuína
Raça	Brahman
Nº de animais machos	35
Nº de animais fêmeas	35
Nº de animais sexo indefinido	0
Peso médio de animais machos	530
Peso médio de animais fêmeas	400
Peso médio de animais sexo indefinido	0
Idade	2 ano(s) e 0 mes(es) e 0 dia(s).
Procedência	Animais de Centrais de Reprodução e Fazendas

Projeto de Pesquisa aprovado em reunião da CEUA em 16/12/2020



JOSÉ NICOLAU PRÓSPERO PUOLI FILHO
Presidente da CEUA da FMVZ, UNESP - Campus de Botucatu