Effects of different variants of the *FASN* gene on production performance and milk fatty acid composition in Holstein × Simmental dairy cows

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Abtract: Crossbreeding of Holstein cattle with bulls of other breeds has garnered increasing interest with respect to efforts to improve performance traits and to decrease problems with fertility and health. Therefore, the objective of this study was to compare the effects of the fatty acid synthase (*FASN*) gene on milk production traits and fatty acid composition of Simmental and first-generation Holstein × Simmental crossbred cows. A total of 105 cows (72 Simmental and 33 crossbred Holstein) were genotyped using the PCR-RFLP method and their fatty acid profiles were analysed. The crossbred Holstein cows with diplotype TW/AR had significantly higher fat content and yield compared to the purebred Simmental cows of the same diplotype. The Holstein × Simmental cows with the diplotype AR/AR were also characterised by significantly lower content of C16:0 and saturated fatty acids, but higher C18:1n9, monounsaturated fatty acid and monounsaturated fatty acid/saturated fatty acid content compared to the same diplotype of the Simmental cattle. These results indicate that with accurate breeding plans, crossbreeding Holstein cows with Simmental bulls could be directed towards a more desirable fatty acid composition of milk and dairy products.

Keywords: milk production; fatty acid synthase; crossbreeding

Cow milk accounts for more than 80% of world milk production and is one of the most common raw materials used for processing (Barlowska et al. 2011). Consequently, milk and dairy products are commonly included in a healthy and balanced diet (Pereira 2014). However, great importance is given to cow milk composition, with emphasis on its fatty acid composition, which could have a major impact on human health (Morris et al. 2007). It is generally accepted that a high proportion of saturated fatty acids, as is found in milk and dairy products, represents a risk factor for cardiovascular

diseases (Markiewicz-Keszycka et al. 2013; Pereira 2014). For instance, myristic (C14:0) and palmitic (C16:0) acids are hypercholesterolaemic and raise concentrations of both low-density lipoprotein and high-density lipoprotein cholesterol (Temme et al. 1997). On the other hand, a diet high in monounsaturated fatty acids is considered favourable, as monounsaturated fatty acids tend to lower plasma cholesterol, low-density lipoprotein and triacylglycerol concentrations (Haug et al. 2007). Oleic acid (C18:1n9), the monounsaturated fatty acid found in milk in the highest proportion, has a positive effect on the concentration of high-density lipoprotein and reduces the concentration of low-density lipoprotein (Temme et al. 1997; Markiewicz-Keszycka et al. 2013). Consequently, fatty acid composition has become an important trait in dairy production. Multiple genes control fatty acid composition; therefore, candidate genes involved in fat synthesis and metabolic pathways could be investigated in order to improve production and milk composition traits (Matsumoto et al. 2012). Fatty acid synthase, a multifunctional enzyme complex that catalyses the *de novo* biosynthesis of long-chain fatty acids, has been mentioned as a promising candidate gene for fat composition of milk and beef (Roy et al. 2006; Schennink et al. 2009; Matsumoto et al. 2012; Li et al. 2016). From 13 identified SNPs (single-nucleotide polymorphisms) on this gene, two non-synonymous SNPs with a potential link to lactation traits were found on exon 34. The A/G substitution at position 5848 was predicted to cause an amino acid substitution from threonine to alanine (T1950A) and the T/C at position 5863 to result in a tryptophan to arginine substitution (W1955R; Matsumoto et al. 2012).

The breeding of cattle is managed to improve the genetic capacity of animals, and ensure that future generations will produce milk in a more efficient way. The mating of genetically similar animals has led to an increase in inbreeding effects which could result in the appearance of undesirable outcomes associated with fertility production, growth and carcass traits (Malchiodi et al. 2014). Therefore, the crossbreeding of dairy cattle has attracted considerable interest in response to concerns regarding fertility and health (Buckley et al. 2014; Puppel et al. 2018).

In the view of the facts mentioned above, the purpose of this study was to compare performance and milk production traits by determining allelic frequencies and identifying the effects of the *FASN* gene on the fatty acid composition of Simmental and first-generation Holstein × Simmental crossbred bulls.

MATERIAL AND METHODS

Animals. A total of 105 milk samples were taken from 72 Simmental and 33 crossbred Holstein (Holstein × Simmental) clinically healthy multiparous (2nd and 3rd lactation) cows from two private farms in Croatia. Information about milk production traits (i.e. milk yield, fat yield, protein yield, fat content and protein content over 305 days) were provided by the Croatian Agricultural Agency, following the regulations of the International Committee for Animal Recording (ICAR 2011). All cows were fed a basal total mixed ratio (TMR) diet (Table 1), consisting of haylage and corn concentrate and had constant access to drinking water. Dry matter intake was not significantly different among

Table 1. Chemical composition of the experimental total mixed ratio diet

NDF, % of dry matter	42.9
ADF, % of dry matter	29.0
Ether extract, % of dry matter	5.6
NEl, Mcal/kg dry matter	1.60
CP, % of dry matter	18.1
RUP, % of dry matter	6.2
RDP, % of dry matter	11.9
Soluble CP, % of dry matter	5.6
Ca, % of dry matter	1.03
P, % of dry matter	0.40
K, % of dry matter	1.54
Mg, % of dry matter	0.32
Na, % of dry matter	0.33
S, % of dry matter	0.18
Cl, % of dry matter	0.49
Mn, mg/kg dry matter	70
Fe, mg/kg dry matter	344
Cu, mg/kg dry matter	25
Zn, mg/kg dry matter	80

ADF = acid detergent fibre; CP = crude protein; NDF = neutral detergent fibre; NEl = net energy of lactation; RDP = ruminally degradable protein; RUP = ruminally undegradable protein

experimental cows. They were milked twice a day. One morning milk sample per cow was collected for DNA isolation and fatty acid analysis.

Genotyping. DNA was isolated from milk samples (350 µl) using the PathoProof™ DNA Extraction Kit (Thermo Fisher Scientific, Finland), following the protocol instructions as described in Thermo Scientific (2015). The primers used in amplification and the PCR reaction were used according to Matsumoto et al. (2012). The PCR products (5 µl) were digested with *Hha* I (T1950A) and *Nci* I (W1955R) restriction enzymes following the supplier's manual. The resulting fragments for both SNPs (T1950A – allele A: one uncut fragment of 336 bp, allele G: two fragments of 262 and 74 bp; W1955R – allele T: one uncut fragment of 336 bp, allele C: two fragments of 247 and 89 bp) were separated on a 3% agarose gel (3 hours/140 V). As the genotypes of T1950A corresponded to those of W1955R, as earlier reported by Matsumoto et al. (2012), they were subsequently analysed as diplotypes (TW/TW, TW/AR, AR/AR).

Milk production parameters and fatty acid composition analysis. Milk quantity, fat and protein content and fatty acid composition were determined. Milk fat extraction and fatty acid analysis were undertaken according to Masek et al. (2014). The composition of each fatty acid was expressed as a percentage of the total fatty acids.

Atherogenicity and thrombogenicity indices. Indices of atherogenicity and thrombogenicity were calculated using the following equations (Ulbright and Southgate 1991):

$$\text{AI} = \frac{\left[(4 \times \text{C14:0}) + \text{C16:0} + \text{C18:0}\right]}{(\text{MUFA} + \text{n6} + \text{n3})} \qquad (1)$$

where:
AI   – atherogenicity index;
C14:0   – myristic acid;
C16:0   – palmitic acid;

C18:0   – stearic acid;
MUFA   – monounsaturated fatty acid;
n3   – n3 fatty acids;
n6   – n6 fatty acids.

$$\text{TI} = \frac{(\text{C14:0} + \text{C16:0} + \text{C18:0})}{(0.5 \times \text{MUFA} + 0.5 \times \text{n6} + 3 \times \text{n3} + \text{n3/n6})} \qquad (2)$$

where:
TI   – thrombogenicity index;
C14:0   – myristic acid;
C16:0   – palmitic acid;
C18:0   – stearic acid;
MUFA   – monounsaturated fatty acid;
n3   – n3 fatty acids;
n6   – n6 fatty acids.

Statistical analysis. Calculation of allele and genotype frequencies, polymorphism deviation from Hardy-Weinberg equilibrium and population genetic indices (observed heterozygosity – H_O, expected heterozygosity – H_E, effective allele number – N_E and fixation index – F_{IS}) were performed using POPGENE32 software, version 1.32 (Yeh et al. 2000). Polymorphism information content (PIC) was calculated according to Botstein et al. (1980). Statistical analyses were performed using Statistica v. 13 (TIBCO Software Inc 2017, USA). The model included breed and genotypes of the *FASN* SNP as fixed effects. Data were assessed for significance using analysis of variance (ANOVA), followed by Tukey's test, in order to determine statistical differences between the group means. Significance was determined at $P < 0.05$.

RESULTS

The allelic frequencies, genotypic frequencies and Hardy-Weinberg equilibrium of the *FASN* gene

Table 2. Allelic frequencies, genotypic frequencies and Hardy-Weinberg equilibrium of the *FASN* gene

Breed	Haplotype	Haplotype frequency	Diplotype	*n*	Diplotype frequency	χ^2	*P*-value
	TW	0.15	TW/TW	1	0.01		
Simmental	AR	0.85	TW/AR	20	0.28	0.32	0.57
			AR/AR	51	0.71		
	TW	0.14	TW/TW	0	0.00		
Holstein Simmental crossbreed	AR	0.86	TW/AR	9	0.27	0.72	0.40
			AR/AR	24	0.73		

are presented in Table 2. The AR haplotype (allele G of SNP T1950A) frequencies observed in this study were 0.85 in Simmental cattle and 0.86 in Holstein Simmental crossbreeds. The most frequently found diplotype was AR/AR in both Simmental (0.71) and crossbred Holstein cattle (0.73), whereas diplotype TW/TW was not found in the crossbreeds (Table 2). Hence, the frequencies of TW haplotype were low in both groups.

The distribution of genotypes was in Hardy-Weinberg equilibrium in both Simmental ($\chi^2 = 0.32$, $P = 0.57$) and crossbred cattle ($\chi^2 = 0.72$, $P = 0.40$; Table 2). In the examined groups, a very slight excess of heterozygosity was found ($F_{IS} = -0.07$ in Simmental cattle and -0.16 in crossbreds; Table 3). This result was also supported by the values of effective allele number (N_E; 1.35 in Simmental cattle and 1.31 in crossbreds; Table 3); in fact, there was an inverse relationship between the value of N_E and the homozygosity of the studied alleles. According to the classification of PIC (PIC value < 0.25 – low polymorphism; 0.25 < PIC value < 0.50 – intermediate polymorphism; and PIC value > 0.50 – high polymorphism), the studied locus possessed low genetic diversity (Table 3).

The effects of the SNPs on milk production traits were analysed and are presented in Table 4. The breed had a significant effect on both fat content and yield ($P = 0.03$ and $P = 0.04$, respectively). Also,

Table 3. Genetic indices

Breed	H_O	H_E	N_E	F_{IS}	PIC
Simmental	0.28	0.26	1.35	−0.07	0.22
Crossbreed	0.27	0.24	1.31	−0.16	0.24

F_{IS} = fixation index; H_E = expected heterozygosity; H_O = observed heterozygosity; N_E = effective allele number; PIC = polymorphism information content

it was found that breed and genotype interaction had a significant effect on fat content ($P = 0.04$). The Holstein Simmental crossbred cows with diplotype TW/AR had significantly higher fat content and yield compared to the Simmental cows of the same diplotype ($P < 0.05$).

Associations between the studied T1950A and W1955R *FASN* polymorphisms and fatty acid composition in Holstein × Simmental crossbreeds are presented in Table 5. Individual fatty acids were significantly influenced by genotype and breed. The content of hypercholesteraemic fatty acid SFA (C14:0 and C16:0) was found to be significantly lower in Holstein crossbred cows. The Holstein crossbred cows with the AR/AR diplotype had a significantly lower content of C16:0 fatty acid compared to the Simmental cattle with the same diplotype ($P < 0.05$). Also, Holstein crossbred cows with the AR/AR diplotype had lower saturated

Table 4. Effect of T1950A and W1955R diplotypes on milk (305d), fat and protein yield and fat and protein content in Simmental cattle and Holstein Simmental crossbreeds. Values are expressed as mean ± standard deviation

Diplotype	Milk (kg) $\overline{x} \pm$ SD	Fat (kg) $\overline{x} \pm$ SD	Fat (%) $\overline{x} \pm$ SD	Protein (kg) $\overline{x} \pm$ SD	Protein (%) $\overline{x} \pm$ SD
Simmental					
TW/TW#	8352.00	305.00	3.65	270.00	3.23
TW/AR	7430.95 ± 1138.09	296.24 ± 40.20[a]	4.01 ± 0.40[a]	254.54 ± 33.93	3.44 ± 0.24
AR/AR	7404.86 ± 1073.26	304.22 ± 48.71	4.11 ± 0.31	258.22 ± 36.10	3.49 ±0.19
Holstein × Simmental crossbreed					
TW/TW#	–	–	–	–	–
TW/AR	7794.48 ± 835.80	347.33 ± 46.95[a]	4.47 ± 0.54[a]	282.13 ± 22.07	3.63 ± 0.20
AR/AR	7501.45 ± 1100.22	308.85 ± 53.47	4.12 ± 0.43	268.41 ± 35.39	3.59 ± 0.24
P-values					
B	0.44	0.04	0.03	0.05	0.02
G	0.59	0.25	0.26	0.60	0.93
B × G	0.65	0.08	0.04	0.36	0.42

B = breed; B × G = breed × genotype; G = genotype
[#]Excluded from further analysis; [a]means with same superscripts within same column differ significantly at $P < 0.05$

fatty acid and higher C18:1n9 and monounsaturated fatty acid than Simmental cows with the same diplotype, and consequently, the AR/AR diplotype showed a higher monounsaturated fatty acid/saturated fatty acid ratio. In contrast, Simmental cows with the TW/AR diplotype had higher C18:1n9 and lower saturated fatty acid content than cows with the AR/AR diplotype. These results show the significant interaction of breed and genotype for the studied traits (P-values of < 0.01, 0.03, 0.01 and < 0.01, respectively). In addition, the content of linoleic acid (C18:2n6*cis*) was significantly influenced by breed ($P < 0.05$). The Holstein crossbred cows had significantly higher values for linoleic acid in comparison to purebred Simmental cattle. The genotype had no statistically significant effect on linoleic acid, in contra to linolenic acid (C18:3n3)

content, which was influenced by genotype but not by breed ($P = 0.03$). However, there were no significant differences between the examined groups. The D9 desaturation index was significantly influenced by breed ($P = 0.02$) and by the interaction of breed and genotype ($P = 0.01$). The Holstein crossbred cows with the AR/AR diplotype had a significantly higher D9 desaturation index compared to the Simmental cattle with the same diplotype ($P < 0.05$). Also, within the Simmental breed, cows with the TW/AR diplotype had a significantly higher D9 desaturation index compared to the cows with the AR/AR diplotype. Regarding the values of atherogenicity index and TI, a significant influence of genotype and breed was observed ($P < 0.05$). The crossbred Holstein × Simmental cows showed lower atherogenicity and thrombogenicity index

Table 5. Effect of T1950A and W1955R diplotypes on fatty acid composition in Simmental cattle and Holstein Simmental crossbreeds. Values are expressed as mean ± standard deviation

FA	Simmental		Holstein Simmental crossbreed		*P*-values		
	TW/AR	AR/AR	TW/AR	AR/AR	B	G	B × G
C4:0	0.91 ± 0.05^a	1.12 ± 0.12^a	1.02 ± 0.01	1.03 ± 0.12	0.75	0.01	0.02
C6:0	1.38 ± 0.38	1.51 ± 0.27	1.42 ± 0.30	1.53 ± 0.51	0.83	0.44	0.97
C8:0	1.02 ± 0.16	1.11 ± 0.08	1.07 ± 0.10	1.07 ± 0.16	0.90	0.47	0.45
C10:0	2.55 ± 0.47	2.78 ± 0.23	2.72 ± 0.15	2.45 ± 0.13	0.55	0.88	0.08
C12:0	3.21 ± 0.71	3.53 ± 0.27^a	3.39 ± 0.25	$2.94 \pm .29^a$	0.30	0.74	0.07
C14:0	11.48 ± 1.33^a	12.55 ± 0.94^a	11.77 ± 0.49	$11.37 \pm .71$	0.32	0.46	0.11
C14:1	0.92 ± 0.27^a	1.23 ± 0.24^a	0.77 ± 0.03	0.96 ± 0.14	0.04	0.01	0.55
C15:0	1.05 ± 0.17^a	1.25 ± 0.17^a	1.08 ± 0.09	1.21 ± 0.14	0.90	0.03	0.65
C16:0	39.23 ± 4.66	40.85 ± 4.53^a	37.50 ± 3.51	34.88 ± 2.65^a	0.04	0.79	0.25
C16:1	2.55 ± 1.15	2.58 ± 1.08	1.88 ± 0.27	2.00 ± 0.32	0.14	0.85	0.91
C17:0	0.44 ± 0.09	0.52 ± 0.07	0.57 ± 0.12	0.63 ± 0.15	0.01	0.11	0.83
C18:0	8.50 ± 1.20^a	9.15 ± 1.16	11.04 ± 1.63^a	9.93 ± 1.84	0.01	0.70	0.14
C18:1n9	20.82 ± 3.95^a	$17.73 \pm 2.46^{a,b}$	21.87 ± 1.61	24.52 ± 0.78^b	< 0.01	0.86	0.03
C18:2n6*cis*	1.09 ± 0.25^a	1.09 ± 0.18	1.36 ± 0.09^a	1.29 ± 0.18	0.01	0.71	0.71
C18:3n3	0.27 ± 0.07	0.29 ± 0.07	0.17 ± 0.13^a	0.33 ± 0.15^a	0.50	0.03	0.11
SFA	70.02 ± 4.02^a	$74.60 \pm 2.87^{a,b}$	71.87 ± 2.70	67.34 ± 1.24^b	0.06	0.98	< 0.01
MUFA	24.29 ± 3.06^a	$21.54 \pm 2.26^{a,b}$	24.51 ± 1.60	27.49 ± 0.89^b	< 0.01	0.92	0.01
MUFA/SFA	0.35 ± 0.07^a	$0.29 \pm 0.04^{a,b}$	0.34 ± 0.04	0.41 ± 0.02^b	0.02	0.88	< 0.01
n6/n3	4.53 ± 2.25	3.99 ± 1.31	6.41 ± 1.78	4.59 ± 2.09	0.16	0.17	0.45
D9*	0.42 ± 0.08^a	$0.35 \pm 0.06^{a,b}$	0.41 ± 0.05	0.49 ± 0.03^b	0.02	0.81	0.01
AI	3.70 ± 0.48	4.47 ± 1.03	4.45 ± 0.37	3.23 ± 0.30	0.71	0.37	0.04
TI	4.25 ± 0.59	5.17 ± 1.28	5.01 ± 0.50	3.58 ± 0.34	0.27	0.44	0.04

$\Delta 9^* = (14:1 + 16:1 + 18:1)/(14:0 + 16:0 + 18:0)$; AI = atherogenicity index; B = breed; B × G = breed × genotype; FA = % from total FA content; G = genotype; MUFA = monounsaturated fatty acid; SFA = saturated fatty acid; TI = thrombogenicity index
[a,b]Means with the same superscripts within the same row differ significantly at $P < 0.05$

for the AR/AR genotype compared with purebred Simmental cattle.

DISCUSSION

There is not a large amount of data on the influence of DNA polymorphisms on milk fatty acid composition, whether in purebred or crossbred cattle. Unlike the data on milk fat percentage and fat yield, data on milk fatty acid composition are not collected routinely or recorded. Therefore, in our study we explored breed and genotypic frequencies for the *FASN* gene and their influence on the fatty acid composition of milk in purebred Simmental and crossbred Holstein × Simmental cows. Both *FASN* mutations observed in this study are non-synonymous SNPs, leading to amino acid changes (T1950A causing a threonine to alanine and substitution and W1955R causing a tryptophan to arginine substitution). Genotypes of T1950A corresponded to those of W1955R in both Simmental cattle and Holstein Simmental crossbreeds. As these results confirm those already reported by Matsumoto et al. (2012) in Holstein cattle, and by Abe et al. (2009) in Japanese Black cattle, it may be suggested that these SNPs are completely linked. In our research, AR was the more frequent haplotype in both groups. Consequently, the TW haplotype frequencies were low in both groups. Similar results for the TW haplotype were reported by Abe et al. (2009) in Holstein (0.171), Angus (0.015) and Hereford (0.071) cattle, while the same authors found a frequency of 0.667 in Japanese Black. The same allele was also found in a frequency of 0.37 (Ciecierska et al. 2013) in the Holstein breed. This wide range of frequencies indicates the great genetic variability of the mentioned SNP in different breeds (Ciecierska et al. 2013).

Regarding the fat content and yield observed in this study, it was evident that breed and interaction of breed and genotype had a significant effect on both traits. The TW/AR diplotype of Holstein crossbreed cows had significantly higher fat content. These results are in accordance with Puppel et al. (2018) who reported that Simmental and Holstein crossbreed cattle had higher concentrations of protein and fat in milk compared with Holstein cattle. A higher protein content and a higher milk yield was reported by Dechow et al. (2007) for crossbreeds of Holstein and Brown Swiss cattle compared with Holstein cows. In contrast, Matsumoto et al. (2012)

reported that the T1950A and W1955R mutations had a significant impact on fat content in Holstein cattle and that the AR/AR diplotype had a higher fat content than the TW/AR diplotype (TW/TW diplotype was excluded from analysis due to an insufficient number of samples). Similar results were reported by Roy et al. (2006). The authors found an association of allele A (from SNP T1950A, resulting in alanine) with increased milk fat content, while animals homozygous for the threonine variant (genotype T/T) were found only in the low-fat group and the allele frequency was low (0.03). In contrast to these findings, Ciecierska et al. (2013) found that first lactation cows with the T/T genotype had higher milk, fat and protein yield than cows with the T/A genotype. From all this, it may be concluded that the association of this *FASN* SNP with milk production traits varies depending on breed and population.

The fatty acid composition of the milk of both groups was significantly influenced by genotype and breed. Our study showed that crossbred cows had significantly lower saturated fatty acid and C16:0. Similarly, effects were also noted on the level of C16:0 in the milk fat of pure Jersey cows and of Jersey × Holstein Friesian crosses, compared to purebred Holstein Friesian cows (Palladino et al. 2010). White et al. (2001) showed that breed significantly affects the content of fatty acids with six to 14 carbon atoms. Likewise, Schennink et al. (2009) reported that the T1950A A/A genotype increased C14:0 and decreased C18:1n9. In contrast to this, Li et al. (2016) reported that in Chinese Holstein cattle the T1950A T/A genotype had higher C14:0 than the A/A genotype. The value for C18:1n9 was higher in our study, and similar results were observed by Puppel et al. (2018) for crossbred Holstein × Simmental cattle. In addition, the levels of linoleic acid in our study (C18:2n6*cis*) were significantly influenced by breed. Holstein × Simmental crossbred cows had significantly higher values for linoleic acid in comparison to purebred Simmental cattle. The genotype had no statistically significant effect on linoleic acid, in contrast to what was observed for linolenic acid (C18:3n3) content, which was influenced by genotype but not by breed. However, there was no significant difference found between the examined groups. These results are not in accordance with those found by other authors (Sasanti 2014; Puppel et al. 2018). The authors of these two earlier studies found higher content of these two essential fatty acids in crossbred cattle. In

our study, the Δ9 desaturation index was influenced by breed and interaction between breed and genotype, but not by genotype. In accordance with this, Schennink et al. (2009) found no influence of the *FASN* gene on the desaturation indices at all, while Matsumoto et al. (2012) reported that they found that the *FASN* gene influenced the C14 desaturation index, but not the C16 and C18 desaturation indices. Dairy products with lower atherogenicity and thrombogenicity index represent potentially healthier food for humans (Puppel et al. 2012). Lower atherogenicity and thrombogenicity index in the AR/AR genotype was observed in crossbred Holstein × Simmental cows in comparison with purebred Simmental cattle.

In this study, fatty acid composition and milk production traits were assessed for the T1950A and W1955R *FASN* polymorphisms in crossbred Holstein × Simmental cattle. Holstein crossbred cows with the AR/AR diplotype had lower saturated fatty acid and higher C18:1n9, C18:2n6*cis*, monounsaturated fatty acid and monounsaturated fatty acid/saturated fatty ratios than Simmental cows with the same diplotype. With the proviso that additional research is needed, the results indicate that the F1 generation of Holstein × Simental crossbreeds expressed better production traits, and this could be useful information for cattle breeding and for the production of milk and dairy products with more desirable fatty acid compositions.

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