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Influences of double interaction effects on main effects and experimental errors in latin square design

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Abstract: *The Latin square design, originally developed by Ronald A. Fisher in 1935, is a classical method for experiments where three factors must be controlled, with one being the factor of interest (treatment) and the other two being nuisance factors. The absence of interactions among these three factors is necessary for the proper use of this design. To investigate the effects of double interactions (treatments vs rows, treatments vs columns, and rows vs columns), 96 Latin squares of order 3 were parametrically constructed, combining 12 randomizations and eight scenarios involving the occurrence or non-occurrence of double interactions. Analysis of variance and the estimated effects of treatments, rows, columns, and experimental errors revealed that the presence of at least one double interaction can alter these effects, as well as the sum of squares of the sources of variation in the experiment. These changes depend on the randomization applied, highlighting the importance of using the Latin square design only in the absence of interactions among the levels of the factors considered in the statistical model.*

Keywords: *LSD, treatment effects; interactions; experimental errors.*

Introduction

Scientific experimentation is one of the fundamental pillars for the advancement of applied sciences such as Agronomy, Animal Science, and Veterinary Medicine, in which the inherent variability of biological systems demands robust experimental strategies. Among the experimental designs widely used to control this variability, the Latin square design (LSD) stands out.

The term *Latin* square was introduced in 18th century by the renowned mathematician Leonhard Euler, and its first application probably occurred in an experiment conducted by the farmer Cretté de Palluel in 1788, aimed at comparing different feeds for sheep, as cited by Freeman (2005).

However, the LSD was formally proposed by Ronald A. Fisher, one of the founders of modern statistics, in his book *The Design of Experiments*, first published in 1935. This design is part of a set of experimental techniques developed to reduce experimental variability and consequently improve statistical precision.

The Latin square design is particularly useful in experiments where three factors must be controlled: one is the factor of interest (treatment) and the other two are nuisance factors (rows and columns) (CRUZ; REGAZZI; CARNEIRO, 2012). According to Montgomery (2020), nuisance factors are those that are expected to influence the response variable but are not of direct interest in the study.

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The LSD is considered a valuable methodological alternative because it allows experiments to be conducted with fewer experimental units without requiring complete homogeneity among them. This occurs because treatments are compared repeatedly across different block combinations, which reduces the impact of local variability.

In the agricultural, animal, and veterinary sciences, the LSD has proven to be an effective strategy for simultaneously controlling two systematic sources of variation. Studies demonstrate its wide application in cultivar evaluation trials, nutritional studies with ruminants, and experiments involving animal diets and management practices (ZIMMERMANN, 2014; FERREIRA, 2018; OLIVEIRA *et al.*, 2020; NASCIMENTO *et al.*, 2023). França *et al.* (2023), when reviewing scientific articles on dairy production, highlighted the frequent use of the LSD in experiments with cattle due to its ability to simultaneously control the effects of animals and experimental periods.

In veterinary medicine, the LSD has also been widely used in clinical studies to evaluate the effectiveness of therapeutic treatments, offering greater control over variations associated with different batches, animals, or environmental conditions (SILVA *et al.*, 2022; SOUZA *et al.*, 2022). However, as pointed out by Lopes and Godoy (2020), an important limitation of the LSD is its inability to directly estimate interactions among treatments, rows, and columns. When such interactions are present, the validity of the results may be compromised, requiring the adoption of more complex statistical models.

In this context, a vast literature discusses the LSD, including important contributions from Cochran and Cox (2005), Gomes (2009) Ryan (2009), Zimmermann (2014), Sampaio (2015), Montgomery (2020) and Ribeiro Júnior (2022). From a statistical perspective, the full model for the LSD, considering one factor of interest (treatment, denoted as τ_i) and two nuisance factors (ω_j and γ_k), can be expressed as follows:

$$y_{ijk}=\mu+\tau_i+\omega_j+\gamma_k+\tau\omega_{ij}+\tau\gamma_{ik}+\omega\gamma_{jk}+\tau\omega\gamma_{ijk},$$

where y_{ijk} is the observed value for the treatment i (i = 1, 2, ..., t), in row j (j = 1, 2, ..., t) and the column k (k = 1, 2, ..., t); μ is the overall population mean; τ_i is the effect of the treatment i; ω_j is the effect of the row j; γ_k is the effect of the column k; $\tau\omega_{ij}$ is the interaction effect between treatment i and row j; $\tau\gamma_{ik}$ is the interaction effect between treatment i and column k; $\omega\gamma_{jk}$ is the interaction effect between row j and column k; and $\tau\omega\gamma_{ijk}$ is the three-way interaction among treatment i, row j, and column k.

When assuming no double or triple interactions, the model simplifies to:

$$y_{ijk}=\mu+\tau_i+\omega_j+\gamma_k$$

However, when it is not possible to assume the absence of interactions effects, the statistical model, under the assumption of additivity, becomes:

$$y_{ijk}=\mu+\tau_i+\omega_j+\gamma_k+\varepsilon_{ijk},$$

where the residual error ε_{ijk} is composed of the double interactions ($\tau\omega_{ij}$, $\tau\gamma_{ik}$, and $\omega\gamma_{jk}$), and the triple interaction ($\tau\omega\gamma_{ijk}$).

Based on this model, in order to test the hypotheses of equality among treatment populations means, or equivalently, the nullity of treatment effects ($\tau_1 = \tau_2 = \ldots = \tau_t = 0$), it is assumed that the residual errors are independent, normally distributed with a population mean of zero, and have the same population variance across treatments. This means that for the assumptions related to the residuals to be satisfied, the interaction effects (double and triple) must behave as random errors: independent, normally distributed, with a mean of zero and constant variance.

Therefore, for the reduced LSD model, composed of both know and unknow effects, to be valid, treatment effects must occur independently of the row and column effects, and the row and column effects must also be independent from each other. Consequently, comparisons of treatment effects can be reliably performed using their marginal means. Otherwise, if at least one significant double or triple interaction is present, confounding between treatment effects and interaction effects may occur, as discussed by Kohli (1988). In such cases, even if the LSD is properly applied, the estimation of at least one main effect may be affected, leading to inaccurate conclusions about its true magnitude.

Given this, the objective of the present study was to evaluate the influence of the double interaction effects $\tau\omega_{ij}$, $\tau\gamma_{ik}$ and $\omega\gamma_{jk}$ on the main effects τ_i, ω_j and γ_k, as well as on the experimental errors obtained in an experiment conducted under the LSD.

Material and Methods

Fatorial Design

The aim of this study was to assess the influence of two-way interactions ($\tau\omega_{ij}$, $\tau\gamma_{ik}$ and $\omega\gamma_{jk}$) on the main effects (τ_i, ω_j and γ_k) and on the error term (ε_{ijk}), which may occur in experiments conducted under a Latin Square Design (LSD). A complete 3x3x3 factorial design was used as a reference. The three levels corresponded to the levels of the factor of interest X (x_1, x_2 and x_3) and the blocking factors B (b_1, b_2 and b_3) and C (c_1, c_2 and c_3), respectively.

The cause-and-effect relationship of the response variable Y with factors X, B and C was represented by a complete fixed-effects factorial model, expressed as:

$$y_{ijk} = \mu + \tau_i + \omega_j + \gamma_k + \tau\omega_{ij} + \tau\gamma_{ik} + \omega\gamma_{jk} + \tau\omega\gamma_{ijk},$$

where y_{ijk} is the observed value for the combination of treatment i (i = 1, 2 and 3), row j (j = 1, 2 and 3) and column k (k = 1, 2 e 3); τ_i, ω_j and γ_k are the main effects of treatment, row, and column, respectively; $\tau\omega_{ij}$, $\tau\gamma_{ik}$, and $\omega\gamma_{jk}$ are the two-way interaction effects between treatment and row, treatment and column, and row and column, respectively; and $\tau\omega\gamma_{ijk}$ is the three-way interaction effect among treatment, row, and column.

The main and interaction effects are defined as deviations from the populations means, such that the sum of the effects within each factor is zero.

The main effects were parameterized as shown in Table 1, which presents the values assigned to treatments, rows, and columns. These values (as well as the interaction effects) were purposely set to be small, allowing for clearer interpretations and greater discrimination of effects in the presence of errors. This parameterization reflects a possible configuration that could occur in an experiment conducted under the LSD.

Table 1: Treatment, row, and column effects.

Effect	Value
τ_1	1
τ_2	2
τ_3	-3
ω_1	-3
ω_2	1
ω_3	2
γ_1	2
γ_2	-3
γ_3	1

Source: from the authors (2025).

The two-way interaction effects $\tau\omega_{ij}$, $\tau\gamma_{ik}$, and $\omega\gamma_{jk}$ were considered under eight distinct scenarios: absence of interactions ($\tau\omega_{ij}=\tau\gamma_{ik}=\omega\gamma_{jk}=0$); only the treatment x row interaction ($|\tau\omega_{ij}|>0$ and the others are null); only the treatment x column interaction ($|\tau\gamma_{ik}|>0$ and the others are null); only the row x column interaction ($|\omega\gamma_{jk}|$ and the others are null); both treatment x row and treatment x column interactions present ($|\tau\omega_{ij}|$ and $|\tau\gamma_{ik}|>0$; $\omega\gamma_{jk}=0$); both treatment x column and row x column interactions present ($|\tau\omega_{ij}|$ and $|\omega\gamma_{jk}|$; $\tau\gamma_{ik}=0$); both treatment x column and row x column interactions present ($|\tau\gamma_{ik}|$ and $|\omega\gamma_{jk}|>0$; $\tau\omega_{ij}=0$); and the presence of all three interactions simultaneously ($|\tau\omega_{ij}|$, $|\tau\gamma_{ik}|$, and $|\omega\gamma_{jk}|>0$). In all scenarios, the three-way interaction was considered null ($\tau\omega\gamma_{ijk}=0$).

The parameterization of the two-way interaction effects $\tau\omega_{ij}$, $\tau\gamma_{ik}$ e $\omega\gamma_{jk}$ was defined as shown in Tables 2, 3, and 4, respectively.

Table 2: Effects of the treatments x rows interaction

	Treatments		
Rows	x_1	x_2	x_3
b_1	$\tau\omega_{11}$ = -3	$\tau\omega_{21}$ = 1	$\tau\omega_{31}$ = 2
b_2	$\tau\omega_{12}$ = 1	$\tau\omega_{22}$ = 2	$\tau\omega_{32}$ = -3
b_3	$\tau\omega_{13}$ = 2	$\tau\omega_{23}$ = -3	$\tau\omega_{33}$ = 1

Source: from the authors (2025).

Table 3: Effects of the treatments x columns interaction.

Treatments	Columns		
	c_1	c_2	c_3
x_1	$\tau\gamma_{11} = 2$	$\tau\gamma_{12} = -3$	$\tau\gamma_{13} = 1$
x_2	$\tau\gamma_{21} = -3$	$\tau\gamma_{22} = 1$	$\tau\gamma_{23} = 2$
x_3	$\tau\gamma_{31} = 1$	$\tau\gamma_{32} = 2$	$\tau\gamma_{33} = -3$

Source: from the authors (2025).

Table 4: Effects of the rows x columns interaction.

Rows	Columns		
	c_1	c_2	c_3
b_1	$\omega\gamma_{11} = 1$	$\omega\gamma_{12} = 2$	$\omega\gamma_{13} = -3$
b_2	$\omega\gamma_{21} = 2$	$\omega\gamma_{22} = -3$	$\omega\gamma_{23} = 1$
b_3	$\omega\gamma_{31} = -3$	$\omega\gamma_{32} = 1$	$\omega\gamma_{33} = 2$

Source: from the authors (2025).

The overall population mean was set to $\mu=0$.

Thus, the 27 response values of Y for each of the eight scenarios were calculated using the equation:

$$y_{ijk}=0+\tau_i+\omega_j+\gamma_k+\tau\omega_{ij}+\tau\gamma_{ik}+\omega\gamma_{jk}+0,$$

for i, j and k = 1, 2 e 3, as presented in Table 5.

Table 5: Observed Y values according to the eight different scenarios of the 3x3x3 Factorial design (continue).

Treat	Ro	Co	A	TR	TC	RC	TRTC	TRRC	TCRC	TRTCRC
x_1	b_1	c_1	0	-3	2	1	-1	-2	3	0
x_1	b_1	c_2	-5	-8	-8	-3	-11	-6	-6	-9
x_1	b_1	c_3	-1	-4	0	-4	-3	-7	-3	-6
x_1	b_2	c_1	4	5	6	6	7	7	8	9
x_1	b_2	c_2	-1	0	-4	-4	-3	-3	-7	-6
x_1	b_2	c_3	3	4	4	4	5	5	5	6
x_1	b_3	c_1	5	7	7	2	9	4	4	6
x_1	b_3	c_2	0	2	-3	1	-1	3	-2	0
x_1	b_3	c_3	4	6	5	6	7	8	7	9
x_2	b_1	c_1	1	2	-2	2	-1	3	-1	0
x_2	b_1	c_2	-4	-3	-3	-2	-2	-1	-1	0
x_2	b_1	c_3	0	1	2	-3	3	-2	-1	0
x_2	b_2	c_1	5	7	2	7	4	9	4	6
x_2	b_2	c_2	0	2	1	-3	3	-1	-2	0
x_2	b_2	c_3	4	6	6	5	8	7	7	9
x_2	b_3	c_1	6	3	3	3	0	0	0	-3
x_2	b_3	c_2	1	-2	2	2	-1	-1	3	0
x_2	b_3	c_3	5	2	7	7	4	4	9	6

x_3	b_1	c_1	-4	-2	-3	-3	-1	-1	-2	0

Table 5: Observed Y values according to the eight different scenarios of the 3x3x3 Factorial design (finish).

Treat	Ro	Co	A	TR	TC	RC	TRTC	TRRC	TCRC	TRTCRC
x_3	b_1	c_2	-9	-7	-7	-7	-5	-5	-5	-3
x_3	b_1	c_3	-5	-3	-8	-8	-6	-6	-11	-9
x_3	b_2	c_1	0	-3	1	2	-2	-1	3	0
x_3	b_2	c_2	-5	-8	-3	-8	-6	-11	-6	-9
x_3	b_2	c_3	-1	-4	-4	0	-7	-3	-3	-6
x_3	b_3	c_1	1	2	2	-2	3	-1	-1	0
x_3	b_3	c_2	-4	-3	-2	-3	-1	-2	-1	0
x_3	b_3	c_3	0	1	-3	2	-2	3	-1	0

Treat: treatment; Ro: row; Co: column; A: absence of interactions; TR: presence of the treatment x row interaction; TC: presence of the treatment x column interaction; RC: presence of the row x column interaction; TRTC: presence of the treatment x row and treatment x column interactions; TRRC: presence of the treatment x row and row x column interactions; TCRC: presence of the treatment x column and row x column interactions; TRTCRC: presence of the treatment x row, treatment x column, and row x column interactions.

Source: from the authors (2025).

Based on these data, the main effects τ_i, ω_j and γ_k were estimated. To illustrate the process, an Analys of Variance (ANOVA) was conducted focusing on the main effects. The interaction effects were incorporated into the error term since the dataset represents the entire population under study (Table 6).

Table 6: Analysis of variance for the 3x3x3 Factorial design.

SV	DF	SS	MS	F value
Treatments	2	$9\sum_{i=1}^{3}\tau_i^2$	$\frac{SSTreat}{2}$	$\frac{MSTreat}{MSRes}$
Rows	2	$9\sum_{j=1}^{3}\omega_j^2$	$\frac{SSRo}{2}$	$\frac{MSRo}{MSRes}$
Columns	2	$9\sum_{k=1}^{3}\gamma_k^2$	$\frac{SSCo}{2}$	$\frac{MSCo}{MSRes}$
Residuals	20	$\sum_{i=1}^{3}\sum_{j=1}^{3}\sum_{k=1}^{3}\varepsilon_{ijk}^2$	$\frac{SSRes}{20}$	
Total	26	$\sum_{i=1}^{3}\sum_{j=1}^{3}\sum_{k=1}^{3}y_{ijk}^2$		

SV: source of variation; DF: degrees of freedom; SS: sum of squares; MS: mean square.

Source: from the authors (2025).

In the 3x3x3 factorial design, the treatment, row, and column effects were correctly obtained. The experimental error was entirely composed of the interactioin effects present in each scenario. Thus, the greater the number of interactions, the higher the residual sum of squares, which consequently reduces the probability of detecting significant main effects.

The residual sums of squares were 0, 126, 252 and 378, corresponding to scenarios with no interactions, one, two, and three two-way interactions, respectively. These values represent 0%, 25%, 40% and 50% of the total sum of squares (Table 7).

Table 7: Treatments, row, and column effects, and Sum of squares (expressed as percentages of the total sum of squares) from the Analysis of variance under the eight different scenarios of the 3x3x3 Factorial design

	A	TR	TC	RC	TRTC	TRRC	TCRC	TRTCRC
t_1	1	1	1	1	1	1	1	1
t_2	2	2	2	2	2	2	2	2
t_3	-3	-3	-3	-3	-3	-3	-3	-3
W_1	-3	-3	-3	-3	-3	-3	-3	-3
W_2	1	1	1	1	1	1	1	1
W_3	2	2	2	2	2	2	2	2
G_1	2	2	2	2	2	2	2	2
G_2	-3	-3	-3	-3	-3	-3	-3	-3
G_3	1	1	1	1	1	1	1	1
SSTreat(%)	33,33	25	25	25	20	20	20	16,67
SSRo (%)	33,33	25	25	25	20	20	20	16,67
SSCo(%)	33,33	25	25	25	20	20	20	16,67
SSRes(%)	0	25	25	25	40	40	40	50

A: absence of interactions; TR: presence of the treatment x row interaction; TC: presence of the treatment x column interaction; RC: presence of the row x column interaction; TRTC: presence of the treatment x row and treatment x column interactions; TRRC: presence of the treatment x row and row x column interactions; TCRC: presence of the treatment x column and row x column interactions; TRTCRC: presence of the treatment x row, treatment x column, and row x column interactions.

Source: from the authors (2025).

Latin Square Design

This study also aimed to evaluate the effects of two-way interactions in a LSD of order 3. This design was chosen for its simplicity of demonstration and because it represents a fraction of the complete 3x3x3 factorial design, using only 9 of the 27 possible combinations of the factors *X*, *B* and *C*. In this context, the values in the LSD vary according to the random assignment of the three treatments to the nine experimental units. For this study, 12 possible randomizations were adopted, as shown in Table 8.

Thus, 9 values were extracted from each of the 96 LSDs of order 3, constructed by considering both the absence of interactions and the presence of at least one two-way interaction, combined with the 12 different randomizations performed.

ANOVA was conducted on these data, and considering the presence of interactions, the model isexpressed as:

$$y_{ijk}=\mu+\tau^*+\omega^*+\Upsilon^*+\varepsilon_{ijk}^*,$$

where y_{ijk} is the observed value for treatment i ($i = 1$, 2 and 3), in row j ($j = 1$, 2 and 3), and column k ($k = 1$, 2 and 3); τ_i^* is the treatment effect, which may incorporate the effect of one or more interactions; ω_j* is the row effect, which may also contain interaction effects; γ_k* is the column effect, likewise subject to interaction effects; and ε_{ijk}^* is the experimental error, which may partially include interaction effects.

Table 8: Twelve different randomizations of treatments x_1, x_2 and x_3 to rows b_1, b_2 and b_3 and to columns c_1, c_2 and c_3 in the Latin square design of order 3

	Randomization 1			Randomization 2			Randomization 3		
	c_1	c_2	c_3	c_1	c_2	c_3	c_1	c_2	c_3
b_1	x_1	x_2	x_3	x_2	x_3	x_1	x_3	x_1	x_2
b_2	x_3	x_1	x_2	x_1	x_2	x_3	x_2	x_3	x_1
b_3	x_2	x_3	x_1	x_3	x_1	x_2	x_1	x_2	x_3
	Randomization 4			Randomization 5			Randomization 6		
	c_1	c_2	c_3	c_1	c_2	c_3	c_1	c_2	c_3
b_1	x_1	x_3	x_2	x_2	x_1	x_3	x_3	x_2	x_1
b_2	x_2	x_1	x_3	x_3	x_2	x_1	x_1	x_3	x_2
b_3	x_3	x_2	x_1	x_1	x_3	x_2	x_2	x_1	x_3
	Randomization 7			Randomization 8			Randomization 9		
	c_1	c_2	c_3	c_1	c_2	c_3	c_1	c_2	c_3
b_1	x_1	x_2	x_3	x_2	x_3	x_1	x_3	x_1	x_2
b_2	x_2	x_3	x_1	x_3	x_1	x_2	x_1	x_2	x_3
b_3	x_3	x_1	x_2	x_1	x_2	x_3	x_2	x_3	x_1
	Randomization 10			Randomization 11			Randomization 12		
	c_1	c_2	c_3	c_1	c_2	c_3	c_1	c_2	c_3
b_1	x_1	x_3	x_2	x_2	x_1	x_3	x_3	x_2	x_1
b_2	x_3	x_2	x_1	x_1	x_3	x_2	x_2	x_1	x_3
b_3	x_2	x_1	x_3	x_3	x_2	x_1	x_1	x_3	x_2

Source: from the authors (2025).

As noted by Kohli (1988), when using the LSD, the main effects may be confounded with interaction effects if these are significant, which can result in inaccurate estimates of treatment effects. Therefore, in the presence of at least one interaction, the parameter estimates from the LSD may incorporate these effects. This makes it impossible to assert that the treatment, row, and column effects are correctly estimated, or that the experimental error consists solely of interaction effects, as is the case in the complete factorial design (Table 7).

The formulas used to calculate the sums of squares in the ANOVA applied to the LSD of order 3 are presented in Table 9.

Table 9: Analysis of variance for the Latin square design of order 3

SV	DF	SS	MS	F value
Treatments	2	$3\sum_{i=1}^{3}\tau_i^{*2}$	$\frac{SSTreat^*}{2}$	$\frac{MSTreat^*}{MSRes^*}$
Rows	2	$3\sum_{j=1}^{3}\omega_j^{*2}$	$\frac{SSRo^*}{2}$	$\frac{MSRo^*}{MSRes^*}$
Columns	2	$3\sum_{k=1}^{3}\gamma_k^{*2}$	$\frac{SSCo^*}{2}$	$\frac{MSCo^*}{MSRes^*}$
Residuals	2	$\sum_{j=1}^{3}\sum_{k=1}^{3}\varepsilon_{ijk}^{*2}$	$\frac{SSRes^*}{2}$	
Total	8	$\sum_{j=1}^{3}\sum_{k=1}^{3}y_{ijk}^{*2}$		

Source: from the authors (2025).

Evaluated Measures

To evaluate the influence of the two-way interactions $\tau\omega_{ij}$, $\tau\gamma_{ik}$ and $\omega\gamma_{jk}$ on the main effects τ_i^*, ω_j^* and γ_k^* manifested in the experiment conducted under the LSD of order 3, the effects of treatments, rows, and columns were obtained. In addition, the sums of squares for treatments (SSTreat*), rows (SSRo*), columns (SSCo*), and residuals (SSRes*) were calculated and expressed as percentages of their respective total sums of squares (SSTotal*) for each LSD.

Based on these values, graphical representations were created for each of the 12 randomizations under the eight different scenarios (ranging from the absence to the simultaneous presence of all three two-way interactions). Additionally, the respective means, maximum and minimum values, and frequency percentages for each individual value were reported.

All measures were calculated separately for the 96 combinations of the 12 randomizations and the eight scenarios of absence or presence of at least one two-way interaction.

Consequently, the closer the results obtained from the LSD of order 3 are to those from the 3x3x3 factorial design, the better. This reflects the theoretical assumption that, since the LSD of order 3 represents one-third of all possible combinations of the three factors, it should, when no interactions are presente, provide results equivalent to those of the complete factorial design.

Results and Discussion

Factorial Design 3x3x3 and Latin Square Design of Order 3

The results obtained in this study indicate that the measures evaluated for the 12 possible randomizations of the LSD of order 3 were identical to those provided by the 3x3x3 factorial design

(Table 7), but only in scenarios where no two-way interactions occurred ($\tau\omega_{ij}$, $\tau\gamma_{ik}$ and $\omega\gamma_{jk}$). This finding reinforces the recommendation by Kohli (1988), who stated that the LSD should only be used when it is assumed that the blocking factors do not interact with each other or with the treatment factor.

When at least one two-way interaction was present, distortions were observed in at least one of the main effects (τ_i, ω_j or γ_k) or in the sums of squares obtained from the ANOVA of the LSD of order 3 or both. This behavior strongly suggests that LSDs of higher orders, which also incorporate two-way and three-way interaction effects into the experimental error, may similarly produce inaccurate values for treatment, row, and column effects.

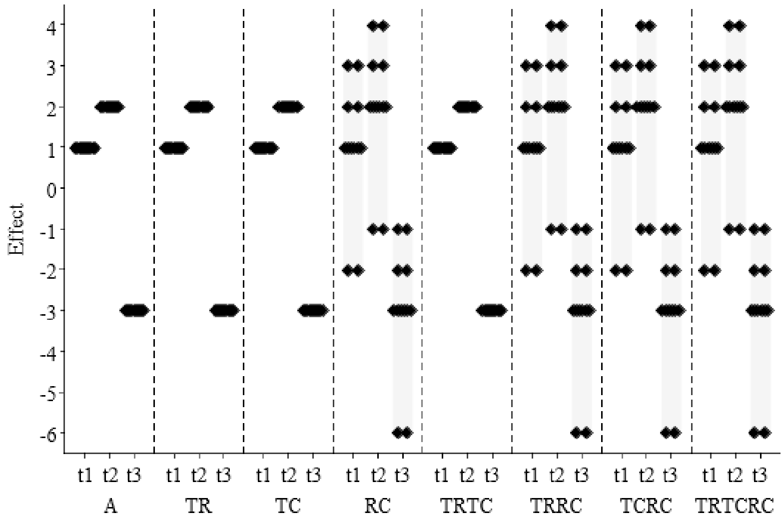
As emphasized by Cox (1958), when such distortions occur in the treatment effects, the conclusions of the experiment remain incomplete until the source of the problem is properly understood. Therefore, any recommendation based on these results becomes questionable. Whenever there is evidence or suspicion of interactions, these should be identified and incorporated into the experimental design to safeguard the validity of the results.

To understand how the LSD behaves in the presence of at least one two-way interaction, all measures were compared to the baseline scenario where no interactions were present (A) in the LSD of order 3.

Main Effects

Since the effects of τ_i, ω_j and γ_k where defined as fixed parameters, varying only according to the respective factor levels, the variation patterns observed for treatment (τ_i*), row (ω_j*), and column (γ_k*) effects were similar, regardless of which main effect was being analyzed. However, these variations occurred due to the influence of the 12 different randomizations, specifically when at least one two-way interaction was present (Figures 1, 2 and 3).

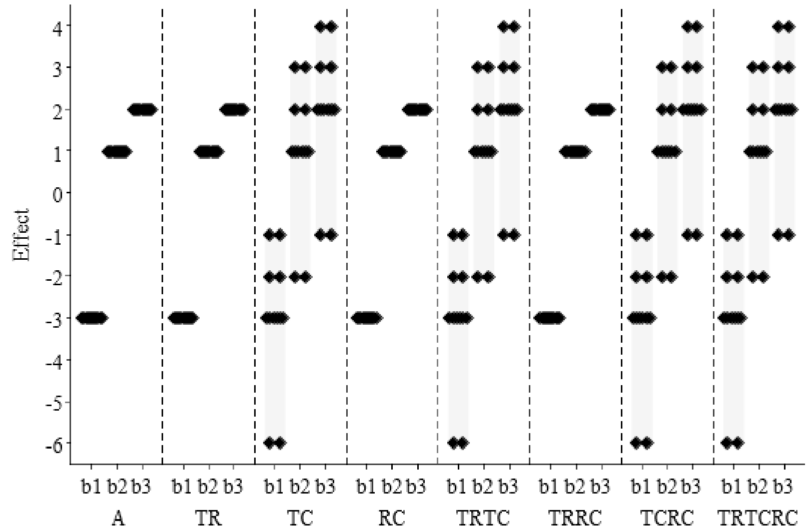
Figure 1: Individual values of treatment effects (t1 τ_1*, t2 = τ_2* and t3 = τ_3*) according to the 12 randomizations of the eight different situations of occurrences of at least one double interaction or absence.



A: absence of interactions; TR: presence of the treatment x row interaction; TC: presence of the treatment x column interaction; RC: presence of the row x column interaction; TRTC: presence of the treatment x row and treatment x column interactions; TRRC: presence of the treatment x row and row x column interactions; TCRC: presence of the treatment x column and row x column interactions; TRTCRC: presence of the treatment x row, treatment x column, and row x column interactions.

Source: from the authors (2025).

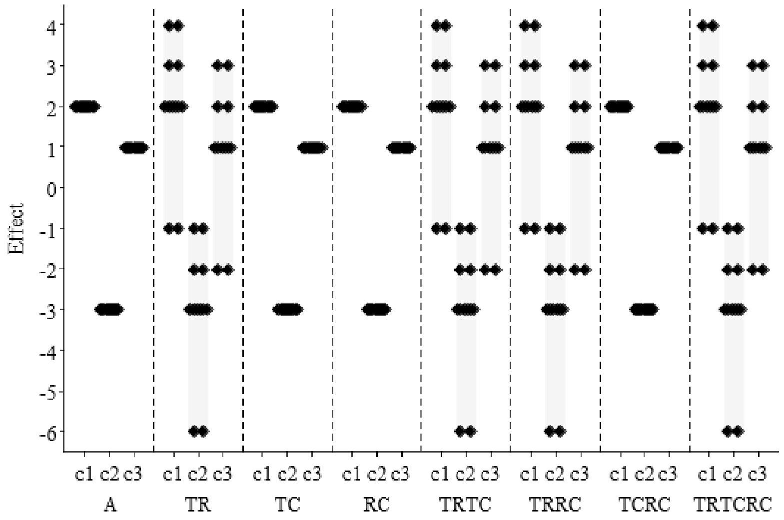
Figure 2: Individual values of raw effects (b1 = ω_1*, b2 = ω_2* and b3 = ω_3*) according to the 12 randomizations of the eight different situations of occurrences of at least one double interaction or absence.



A: absence of interactions; TR: presence of the treatment x row interaction; TC: presence of the treatment x column interaction; RC: presence of the row x column interaction; TRTC: presence of the treatment x row and treatment x column interactions; TRRC: presence of the treatment x row and row x column interactions; TCRC: presence of the treatment x column and row x column interactions; TRTCRC: presence of the treatment x row, treatment x column, and row x column interactions.

Source: from the authors (2025).

Figure 3: Individual values of column effects (c1 = γ_1*, c2 = γ_2* and c3 = γ_3*) according to the 12 randomizations of the eight different situations of occurrences of at least one double interaction or absence.



A: absence of interactions; TR: presence of the treatment x row interaction; TC: presence of the treatment x column interaction; RC: presence of the row x column interaction; TRTC: presence of the treatment x row and treatment x column interactions; TRRC: presence of the treatment x row and row x column interactions; TCRC: presence of the treatment x column and row x column interactions; TRTCRC: presence of the treatment x row, treatment x column, and row x column interactions.

Source: from the authors (2025).

These deviations were directly related to the type of interaction. For treatment effects (τ_1*, τ_2* and τ_3*), inconsistencies were evident in the RC, TRRC, TCRC, and TRTCRC scenarios (Figure 1). Row effects (ω_1*, ω_2* and ω_3*) varied under TC, TRTC, TCRC, e TRTCRC (Figure 2). Column effects (γ_1*, γ_2* and γ_3*) were altered in TR, TRTC, TRRC, e TRTCRC (Figure 3.3).

These results show that when at least one two-way interaction is present, the value of at least one main effect does not represent its true magnitude, as it becomes directly influenced by the interaction. Specifically, treatment effects were altered when a row-by-column interaction occurred (Figure 1); row effects were affected by a treatment-by-column interaction (Figure 2); and column effects were impacted by a treatment-by-row interaction (Figure 3). Furthermore, the magnitude of this influence depends on the randomization adopted in the LSD of order 3.

Another important point is that the correct ranking of the treatment effects ($\tau_2 > \tau_1 > \tau_3$), row effects ($\omega_3 > \omega_2 > \omega_1$) and column effects ($\gamma_1 > \gamma_3 > \gamma_2$) may be compromised when at least one two-way interaction is present. This occurs because the adjusted values for treatments (τ_1*, τ_2* and τ_3*), rows (ω_1*, ω_2* and ω_3*) and columns (γ_1* $\gamma_2*$and γ_3*)become less distinct (Figures 3.1, 3.2, and 3.3).

On the other hand, the treatment effects retained their correct values ($\tau_1* = \tau_1*$, $\tau_2* = \tau_2*$ and $\tau_3* = \tau_3*$) in scenarios A, TR, TC, and TRTC, that is, whenever the row-by-column interaction was absent (Figure 1).

A similar pattern was observed for row and column effects. Row values remained accurate ($\omega_1* = \omega_1$, $\omega_2* = \omega_2$ and $\omega_3* = \omega_3$) in scenarios A, TR, RC, e TRRC, meaning when the treatment-by-column interaction was not present (Figure 2). Column values were correct ($\gamma_{¿}* = \gamma_1$, $\gamma_2* = \gamma_2$ and $\gamma_3* = \gamma_3$) in scenarios A, TC, RC, e TCRC, that is, when there was no treatment-by-row interaction (Figure 3).

The frequency analysis of the treatment values obtained from the 96 LSDs of order 3 (Table 1) revealed that in 75% of the cases, the treatment values corresponded perfectly to their true values ($\tau_1* = 1$, $\tau_2* = 2$ and $\tau_3* = -3$). However, in 8.33% of the cases, the values were lower than expected, and in 16,67%, they were higher than the actual values.

Even though the treatment effects were correct in most cases, this is not sufficient to justify the use of the LSD when a row-by-column interaction is present. This is because the primary goal of an experiment is not only to obtain correct numerical values but also to detect whether meaningful differences exist among the treatments. For that purpose, the correct decomposition of variability into sums of squares is essential.

Table 10: Frequencies (%) of observed treatment effects patterns

Effect	Frequencie		
	τ_1*	τ_2*	τ_3*
- 6	0	0	8,33
- 3	0	0	75
- 2	8,33	0	8,33
- 1	0	8,33	8,33
1	75	0	0
2	8,33	75	0
3	8,33	8,33	0
4	0	8,33	0

Source: from the authors (2025).

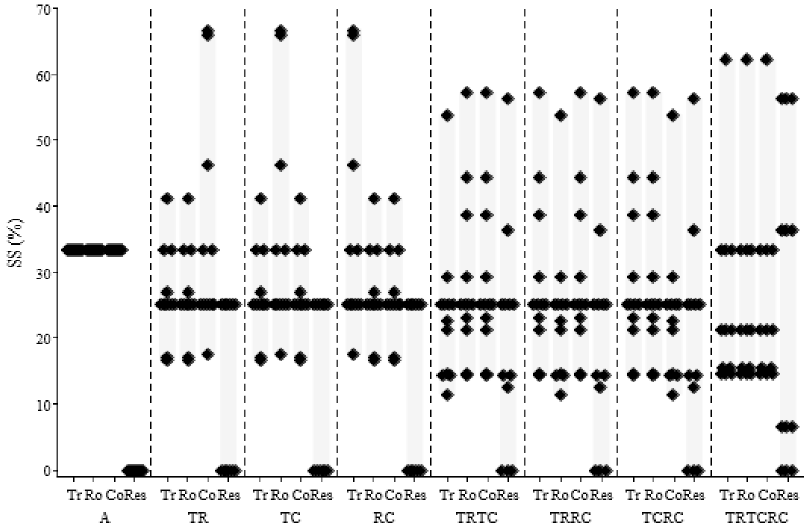
Sum of Squares

The sums of squares for treatments (SSTreat*), rows (SSRo*), columns (SSCo*), and residual (SSRes*) obtained from the ANOVA of the LSD of order 3 were compromised whenever at least one two-way interaction was present. In these situations, the interaction effects were absorbed, either fully or partially, into the residual, altering the distribution of all sums of squares (Figure 3.4)

In absence of two-way interactions, all sums of square, expressed as percentages, remained constant and identical across trandomizations, with SSTreat* = SSRo* = SSCo* = 33.33% and SSRes* = 0% (Figure 4).

However, even as SSRes* increased, SSTreat*, SSRo*, and SSCol* could remain relatively high depending on the type of interaction. Specifically, the presence of row-by-column, treatment-by-column, or treatment-by-row interactioins directly affected SSTreat*, SSRo*, and SSCo*, respectively. When at least two two-way interactions occurred, the values of the three primary sums of squares approached the value of SSRes*, depending on the randomization. In these conditions, even when actual differences existed among treatment, row, or column effects, the LSD of order 3 could fail to detect them statistically (Figure 4).

Figure 4: Sum of squares of treatments (Tr), rows (Ro), columns (Co), and residual (Res), as a percentage o their respective total sum of squares, according to the 12 randomizations of the eight different situations of at least one double interaction occurrence or not.



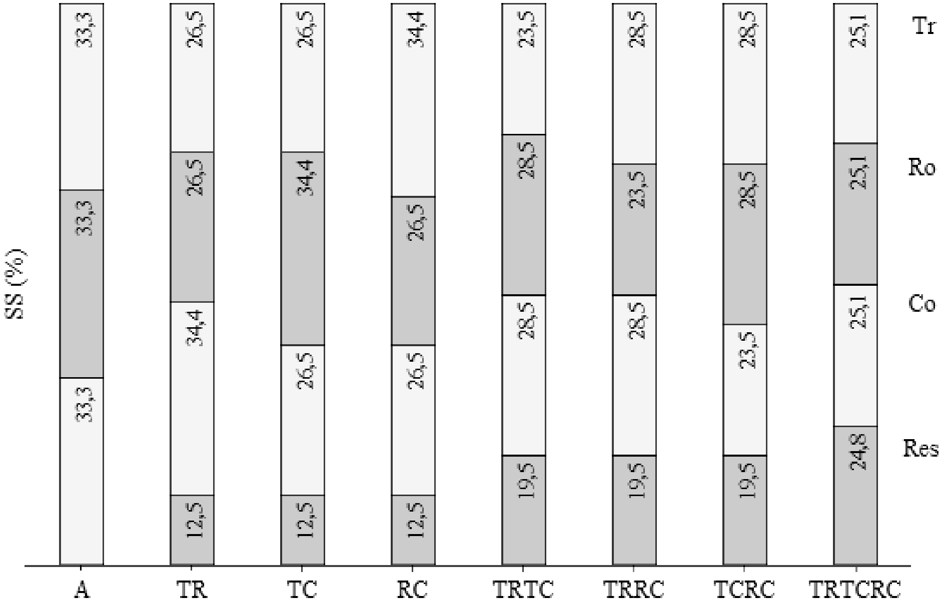
A: absence of interactions; TR: presence of the treatment x row interaction; TC: presence of the treatment x column interaction; RC: presence of the row x column interaction; TRTC: presence of the treatment x row and treatment x column interactions; TRRC: presence of the treatment x row and row x column interactions; TCRC: presence of the treatment x column and row x column interactions; TRTCRC: presence of the treatment x row, treatment x column, and row x column interactions.

Source: from the authors (2025).

As shown, the average SSREs* increased progressively with the number of two-way interactions, from 0 (scenario A), to 12.50% (TR, TC, e RC), then to 19.47% (TRTC, TRRC, e TCRC), and finally to 24.82% (TRTCRC). At the same time, SSTreat*, SSRo*, and SSCo* decreased, particularly in TRTCRC (Figure 5).

Overall, in the LSD of order 3, the presence of at least one two-way interaction leads to confounding in at least one main effect (treatment, row, or column) depending on the randomization used. Part of the interaction effect shifts into the residual, compromising the proper decompostition of variability. When this happens, it indicates that some portion of the interaction has not been fully accounted for within the error term. This is reflected not only by the increase in SSRes* but also by the fluctuations in SSTreat*, SSRo*, and SSCo*.

Figure 5: Means of the sums of squares for treatments (Tr), rows (Ro), columns (Co), and the residue (Res), as a percentage of the respective total sums of squares, according to the 12 randomizations of the eight different situations of at least one double interaction occurrence or not.



A: absence of interactions; TR: presence of the treatment x row interaction; TC: presence of the treatment x column interaction; RC: presence of the row x column interaction; TRTC: presence of the treatment x row and treatment x column interactions; TRRC: presence of the treatment x row and row x column interactions; TCRC: presence of the treatment x column and row x column interactions; TRTCRC: presence of the treatment x row, treatment x column, and row x column interactions.

Source: from the authors (2025).

This pattern suggests that LSDs of higher orders may behave similarly in the presence of two-way interactions.

Although the results varied according to the randomization used, searching for a specific treatment allocation that minimizes these distortions is not recommended, since randomization is a fundamental principle of experimental design and must not be violated.

In line with the results presented, Ribeiro (2019) also concluded that the treatment effects are frequently affected in the presence of interactions. Furthermore, experimental precision is reduced because interaction effects are incorporated into the residual, which decreases the statistical significance of treatments. It is important to emphasize that this issue arises primarily from the inadequate adoption of the statistical model rather than from the influence of uncontrollable factors themselves.

Therefor, the classical statistical model for LSD, represented by:

$$y_{ijk}=\mu+\tau_i+\omega_j+\gamma_k+\varepsilon_{ijk},$$

is only valid when the complete absence of interactions is assured.

If any interactioin exists, the use of the LSD is not recommended. As demonstrated for the LSD of order 3, and likely applicable to higher-order designs, the cause-effect relatioinship between the factor of interest (X), the blocking factors (B and C), and the response variable (Y) is better represente by:

$$Y_{ijk} = \mu + \tau_i^* + \omega_j^* + \gamma_k^* + \varepsilon_{ijk}^*.$$

Conclusion

The presence of at least one two-way interaction (treatment x row, treatment x column, or row x column) can alter the values of the main effects (treatment, row, and column) as well as the experimental error when using a LSD of order 3.

These interactions also affect the sums of squares for the sources of variation considered in the analysis of variance. As a result, an effect that would otherwise be statistically significant may be incorrectly interpreted as non-significant, compromising the validity of the experiment's conclusions

Given these results, the use of the LSD of order 3 is recommended only when there is strong confidence that no interaction effects exist between the factors involved. Otherwise, this design is not advisable, and it is preferable to adopt alternative experimental designs that can properly account for such interactions within the statistical model.

Although this study focused solely on the LSD of order 3, based on the theoretical foundation that the experimental error in the classical LSD model inherently incorporates the effects of two-way and three-way interactions, it is reasonable to expect that similar conclusions apply to LSDs of higher orders.

In summary, this study reinforces that the correct specification of the statistical model is essential to ensure the validity of inferences when using the LSD. The application of this design must be carefully conditioned on verifying the absence of meaningful interactions among the factors. Failure to do so may compromise both the accuracy of the results and the reliability of the experimental conclusions.

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