Spanish Journal of Agricultural Research
17 (4), e0505, 11 pages (2019)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2019174-15316
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Evaluation and cluster analysis of inflammatory reactions of dairy cattle mastitis pathogens in milk samples submitted for microbiological examination

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Abstract

Aim of study: We have classified into homogenous groups a wide spectrum of mammary pathogens according to their frequency of isolation in clinical mastitis and their somatic cell counts in mastitis.

Area of study: The study was conducted in Galicia (NW Spain).

Material and methods: 163,741 dairy cattle quarter milk samples were analyzed. We identified mastitis pathogens to the species level and performed a cluster analysis to classify these microorganisms by their median of Linear Score (mLS), percentage of isolation in clinical mastitis samples (%ICS) and percentage of isolation in samples with somatic cell counts under 100,000 cells/mL (%ISU100).

Main results: Forty-three different species were isolated. Cluster analysis identified 4 groups of pathogens; mLS and %ICS progressively increased from Group I to Group IV and %ISU100 decreased. However, several pathogens included in groups II and III showed %ISU100 values higher than 2% and 3%. Minor pathogens were mainly clustered in Group I (e.g., *Corynebacterium* spp. and most of *Staphylococcus* spp.), while known major pathogens were included in Groups II, III y IV. Species of the same family, genus or microbiological group like *Enterobacteriaceae* or *Enterococcus* spp. were frequently separated into different groups, thus showing heterogeneity among the members of these groups.

Research highlights: Results obtained here may aid in assessing the pathogenicity of sporadic pathogens in relation to more well-known pathogens and suggest that the traditional classification between minor and major pathogens is an oversimplification of the reality, especially for the latter category.

Additional keywords: subclinical mastitis; clinical mastitis; major pathogens; minor pathogens; somatic cell counts.

Abbreviations used: %ICS (percentage of isolation in clinical mastitis samples); %ISU100 (percentage of isolation in samples with somatic cell counts under 100,000 cells/mL); IMI (intramammary infection); LS (linear score); mLS (median of linear score); NAS (non-*aureus* staphylococci); PCA (principal components analysis); SCC (somatic cell counts); SP (similarity profile).

Authors' contributions: Conceived and designed the work: GF. Performed the experiments and acquisition of data: MLB, BP and RL. Statistical analysis: JMD. Interpretation of data JMD, JMA and GF. Critical revision of the manuscript for important intellectual content: CLN, AP, AI and PDB. Wrote the paper: JMD and GF.

Citation: Díaz-Cao, JM; Barreal, ML; Pombo, B; Prieto, A; Alonso, JM; Iglesias, A; Lorenzana, R; López-Novo, C; Díez-Baños, P; Fernández, G (2019). Evaluation and cluster analysis of inflammatory reactions of dairy cattle mastitis pathogens in milk samples submitted for microbiological examination. Spanish Journal of Agricultural Research, Volume 17, Issue 4, e0505. https://doi.org/10.5424/sjar/2019174-15316

Received: 19 Jun 2019. **Accepted:** 08 Jan 2020.

Funding agencies/Institutions	Project / Grant
Xunta de Galicia, Program for consolidating and structuring competitive research groups	GRC2015/003

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Bovine mastitis is a multietiologic disease that can be induced by more than 130 different species of microorganisms. Mastitis pathogens have traditionally been classified as "minor" or "major" pathogens in order to assess the prognosis of the infection and to establish priorities in the adoption of preventive and

control measures. Thus, major pathogens are related to a higher impact on cattle udder health, as well as on milk quality and productivity (Zadoks & Fitzpatrick, 2009).

This classification has generally been based on the frequency of presentation in clinical mastitis as well as on the somatic cell counts (SCC) in subclinical mastitis, since it is broadly considered that the existence of an intramammary infection (IMI) is the most important cause of an SCC increase (Dohoo & Meek, 1982). Different thresholds have been set to SCC to consider the existence of IMI in subclinical mastitis (100,000–500,000 cells/mL), but 200,000 cells/mL is the most widely used (Schwarz *et al.*, 2010; IDF, 2013). However, since SCC also vary with the lactation stage, age, stress of the animals, season and time and frequency of milking (Dohoo & Meek, 1982; Harmon, 1994), this threshold may inaccurately classify some mastitis (Schwarz *et al.*, 2010).

Some microorganisms like *Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Staphylococcus aureus, Escherichia coli* or *Klebsiella pneumoniae* are generally considered in literature as major pathogens (Bradley, 2002; Zadoks & Fitzpatrick, 2009), whereas coagulase-negative staphylococci and *Corynebacterium* spp. are considered minor pathogens (Bradley, 2002; Pyörälä & Taponen, 2009). However, there are a number of pathogens which regularly present low rates of isolation (Pitkälä *et al.*, 2004; Tenhagen *et al.*, 2006) and have not been reported in any of those groups (e.g., *Streptococcus* spp. or *Enterococcus* spp., as well as yeasts, coliforms or other Gram-negatives, etc.) (Bradley, 2002). Consequently, when these pathogens are studied, they are frequently merged in groups with other bacteria on the basis of genus, family or microbiological group. Thus, their effects on cattle mastitis are considered as a group or assumed to be similar to other more well-known pathogens of the same genus or microbiological group (Djabri *et al.*, 2002; de Haas *et al.*, 2004; Malinowski *et al.*, 2006; Schwarz *et al.*, 2010). Nevertheless, these groups may not be so homogenous to make such assumptions. For example, non-*aureus* staphylococci (NAS) are historically studied as a group in cattle mastitis and, while different authors agree that SCC in IMI caused by NAS as a whole are lower than that when caused by *Staph. aureus* (Schukken *et al.*, 2009; Supré *et al.*, 2011; Taponen *et al.*, 2007), results are contradictory when different NAS species are individually compared. Therefore, several authors did not find any difference among NAS regarding the severity of clinical signs (Jarp, 1991; Taponen *et al.*, 2006) or SCC (Thorberg *et al.*, 2009), while others did find statistically significant differences (Sampimon *et al.*, 2009; Fry *et al.*, 2014; Condas *et al.*, 2017), and some NAS species (e,g. *Staphylococcus chromogenes, Staphylococcus simulans* and *Staphylococcus xylosus*) even presented similar SCC to *Staph. aureus* (Supré *et al.*, 2011).

Pathogens with low rates of isolation in mastitis gain importance by summing all the cases in which they all are involved (Makovec & Ruegg, 2003; Piepers *et al.*, 2007; Schwarz *et al.*, 2010; Kalmus *et al.*, 2011). Besides, mastitis control programs have decreased the prevalence of some pathogens, such as *Staph. aureus* or *Strep. agalactiae*, but also increased the relative rate of isolation of others, which were less frequent in the past (Zadoks & Fitzpatrick, 2009; Fernández *et al.*, 2013). Consequently, it is necessary to gain solid knowledge about the relative behavior of these pathogens in cattle mastitis.

Thus, the aim of this study was to analyze the behavior of a wide spectrum of mammary pathogens regarding different variables: percentage of isolation in clinical mastitis, SCC in non-clinical mastitis and percentage of isolation in samples with SCC ≤ 100, 000 cells/mL. These results were subsequently used to classify the different mammary pathogens into homogenous groups.

Material and methods

Study area, collection and processing of samples

Galicia (NW Spain) is the largest cattle-rearing region in Spain, accounting for 53% of Spain's dairy farms and approximately 350,000 dairy cows older than two years of age. This region accounts for 35% of the milk production in Spain, that is around 1.7% of the milk produced in the European Union.

The study included all quarter milk samples submitted to the *Laboratorio Interprofesional Gallego de Análisis de Leche* (LIGAL) for microbiological examination from June 2005 to September 2011. Milk samples were collected by farm personnel and veterinarians, following the instructions of the Galician mastitis control program. They were submitted when farmers detected clinical mastitis or when practitioners detected a SCC increase in bulk-tank.

Milk samples were analyzed by spreading 10 µL of milk on a 5% sheep blood agar plate. Plates were incubated aerobically at 37 ºC and read at 24 h and 48 h. Plates which showed no growth at 48 h, were kept in incubation for 3 more days in which daily readings were performed.

An IMI was stated for *Staph. aureus* or *Strep. agalactiae* when at least 100 cfu/mL were isolated (Dohoo *et al.*, 2011). For the rest of the microorganisms,

a minimum of 500 cfu/mL were required to consider IMI (Taponen *et al.*, 2006; Thorberg *et al.*, 2009; Nam *et al.*, 2010; Dohoo *et al.*, 2011). Plates with no growth and primary culture plates containing more than one colony type were removed from the study.

Isolates were preliminary identified by colony morphology, hemolytic patterns, Gram-staining and catalase-test. Subsequently, identification was completed using the VITEK 2 system (bioMérieux, Hazelwood, MO, USA) with the VITEK 2 cards GP-test kit (for Gram-positive cocci), GN-test kit (for Gram-negative) and YST test kit (for yeasts and *Prothoteca* spp.). The procedures recommended by the manufacturer were followed and bacterial identification was regarded as acceptable only when the confidence level of the analysis was > 85%. However, three exceptions were considered: *Corynebacterium* spp., *Nocardia* spp and *Trueperella pyogenes*. In these cases, the identification was based on characteristics of isolation on blood-agar plates: form, presence of hemolysis and time of growth; Gram-staining and the result of the catalase test, following the characteristics defined for the identification of these genera and species (Brown *et al.*, 1981; Quinn *et al.*, 2011).

The isolates included in the analysis were identified to the species level, except in the case of *Lactococcus* spp., *Corynebacterium* spp., *Nocardia* spp. and *Prototheca* spp.

Definitions used in this study

Milk samples were classified as clinical mastitis when a visible alteration in the milk or udder was observed in the animal by farm veterinarians; otherwise, they were considered non-clinical mastitis. Somatic cell counts were performed in all the non-clinical mastitis samples using a Fossomatic-FC somatic cell counter (Foss, Denmark) and following the normalized protocol ISO 13366-2/IDF 148-2 accredited by the National Entity of Accreditation (ENAC). Non-clinical mastitis samples were additionally divided into two groups: SCC ≤ 100,000 cells/mL and SCC > 100,000 cells/mL, thus defining this value as a cut-off, because the inflammatory reaction is thought to start at this level (Schwarz *et al.*, 2010).

In some cases, SCC could not be performed due to the scarce quantity of milk of the sample. Consequently, these samples were classified as insufficient and removed from the study.

Statistical analysis

The statistical analysis was performed in samples which met the following criteria: a unique pathogen was isolated and the proportion of samples in which this pathogen was isolated was over 0.1%. Somatic cell count values in samples with SCC > 100,000 cells/ mL were transformed to Linear Score (LS) using the recommended formula by Shook (1982):

$$LS = \log_2 (SCC/100) + 3$$

The statistical analysis was performed with the statistical software R (R Core Team, 2014). The values of the median of LS (mLS), percentage of isolations in clinical mastitis (%ICS) and percentage of isolations in samples with SCC ≤ 100,000 cell/mL (%ISU100) regarding the total samples in which each microorganism was isolated were obtained for each microorganism and used to perform a cluster analysis. Firstly, values of these variables were z-standardized and a principal component analysis (PCA) was performed to explore the linear relationship between the variables. Subsequently, a hierarchical cluster analysis was performed according to the Ward's method (Ward, 1963) from the three principal components obtained. PCA and cluster analysis were performed using the function "HCPC" in the package "FactoMineR" (Lê *et al.*, 2008; Husson *et al.*, 2010). This function also performs the V-test which allows to estimate the presence of a significant overexpression (if > 2) or infraexpression (if < -2) of the variables in the resulting clusters (Lê *et al.*, 2008; Husson *et al.*, 2010). The significance of the obtained clusters was assessed by a Similarity Profile (SP) analysis with the assumption of no "*a priori*" groups using the function "simprof" from the "clustsig" package (Clarke *et al.*, 2008). The analysis was performed setting 10,000 similarity profiles for the expected distribution of the data and 9,999 for the comparison with its null distribution.

Results

A total of 240,232 samples were submitted to the laboratory during the period of study; 32,852 (13.7%) were contaminated, 30,561 (12.7%) did not show any growth, and more than one species were isolated in 1,854 (0.8%) samples. Consequently, these samples were removed from the study. Additionally, 1,620 were also removed because the quantity of the sample was insufficient. As a result, 173,345 samples were finally included in this study; 50,389 (29.1%) were classified as clinical mastitis and 122,956 (70.9%) as non-clinical. Among the samples categorized as non-clinical mastitis, 8,036 (6.6%) presented SCC ≤ 100,000 cells/mL.

Forty-three different species were identified. However, 5.4% of Gram-positive catalase-positive cocci

could not be identified to the species level, as well as 1.9% of *Streptococcus* spp. different from *Strep. agalactiae*, 2.2% of *Enterococcus* spp. and 1.3% of Gram-negative. In 20% of yeasts, the identification to, at least the genus level, could not be achieved either.

Pathogens whose isolation frequency was over 0.1% accounted for a total of 163,741 (94.5%) samples. They represented 94.2% of the non-clinical samples with SCC > 100,000 cells/mL (n = 108,226/114,889), 95% of the non-clinical samples with SCC ≤ 100,000 cells/mL (n = 7,714/8,120), and 94.8% of the clinical samples (n = 47,719/50,336). The SCC and LS values of these pathogens are presented in Table 1, which shows data from the SCC > 100,000 threshold. Table 2 shows the %ICS and %ISU100 for each microorganism identified.

Principal component analysis prior to clustering provided one first component (Dim 1) which accounted for 87.46% of the variance. The observed variables contributed to this component similarly (30.84%-36.15%), being positively correlated in the case of mLS and %ICS and negatively in %ISU100. The second component (Dim 2) accounted for a 10.09% of the variability, highlighting the contribution of %ICS (61.05%) and, to a lesser extent, %ISU100 (36.97%), although the correlations were low (Table 3).

Figure 1 shows the dendrogram from the cluster analysis. Looking at the results in the dendrogram, the optimum solution was considered when a cut-off threshold at a height of 3.88 was selected, resulting in the existence of four groups. The distribution of these clusters according to the coordinates of the microorganisms in the two main components is shown in Fig. 2a. Pairwise plots between the observed values of the three variables (mLS, %ICS and %ISU100) can be observed in Figs. 2b, 2c and 2d. In general, there is a good agreement between the distribution of the microorganisms and the assigned clusters, especially, between %ICS and %ISU100 (Fig. 2d).

Table 1. Mean and median of the Linear Score (LS) and somatic cell counts (× 1,000 cells/mL) of each microorganism identified in dairy cattle milk samples from Galicia (NW Spain) with a percentage of isolation over 0.1% in samples with somatic cell counts (SCC) > 100,000 cells/mL.

Microorganism	Nº	LS				SCC (× 1,000 cell/mL)			
		mean	SEM	median	SD	mean	SEM	median	SD
Aerococcus viridans	281	6.39	0.105	6.32	1.76	2,131	0.131	1,373	2.19
Candida famata	417	7.60	0.071	7.64	1.45	3,787	0.177	2,493	3.61
Candida koseri	235	7.28	0.103	7.29	1.58	3,293	0.225	1,957	3.45
Candida krusei	407	8.29	0.065	8.37	1.31	5,626	0.238	4,145	4.80
Candida rugosa	1,051	7.92	0.039	7.94	1.27	4,366	0.123	3,072	3.99
Candida tropicalis	323	7.78	0.080	7.64	1.43	4,344	0.237	2,488	4.26
Corynebacterium spp.	21,688	6.11	0.012	5.99	1.81	1,926	0.020	793	2.94
Enterobacter cloacae	297	7.08	0.109	7.20	1.88	3,544	0.258	1,845	4.44
Enterococcus faecalis	3,650	6.50	0.028	6.42	1.70	2,221	0.050	1,072	3.03
Enterococcus faecium	1,276	7.81	0.037	7.93	1.32	4,038	0.101	3,048	3.60
Enterococcus saccharolyticus	659	7.78	0.058	7.91	1.49	4,290	0.158	3,012	4.06
Escherichia coli	4,771	7.97	0.026	8.25	1.77	5,617	0.079	3,795	5.44
Klebsiella oxytoca	375	7.47	0.093	7.67	1.80	4,133	0.227	2,549	4.39
Klebsiella pneumoniae	1,035	7.97	0.052	8.15	1.68	5,398	0.163	3,539	5.23
Lactococcus spp.	2,891	7.11	0.032	7.20	1.72	3,209	0.069	1,845	3.73
Nocardia spp.	163	9.28	0.060	9.31	0.77	8,825	0.340	7,953	4.34
Pasteurella multocida	111	8.87	0.127	9.27	1.34	7,895	0.471	7,719	4.96
Prototheca spp.	833	8.28	0.037	8.38	1.07	4,923	0.117	4,164	3.39
Pseudomonas aeruginosa	201	7.70	0.124	7.89	1.76	4,629	0.319	2,970	4.52
Raoultella ornithinolytica	255	6.72	0.117	6.96	1.87	2,676	0.194	1,551	3.09
Serratia liquefaciens	344	6.69	0.104	6.66	1.92	2,834	0.198	1,266	3.67
Serratia marcescens	545	7.85	0.066	7.95	1.53	4,619	0.188	3,088	4.38
Staphylococcus aureus	20,441	7.22	0.012	7.29	1.69	3,418	0.027	1,954	3.89
Staphylococcus chromogenes	2,781	6.17	0.035	6.05	1.82	1,994	0.056	826	2.95
Staphylococcus epidermidis	2,741	6.38	0.033	6.31	1.75	2,113	0.054	991	2.85

Table 1. Continued.

Microorganism	Nº	LS				SCC (× 1,000 cell/mL)			
		mean	SEM	median	SD	mean	SEM	median	SD
Staphylococcus gallinarum	229	6.36	0.114	6.41	1.73	2,020	0.167	1,064	2.53
Staphylococcus haemolyticus	1,903	6.39	0.041	6.33	1.79	2,197	0.071	1,005	3.08
Staphylococcus hominis	348	5.83	0.084	5.76	1.56	1,308	0.102	675	1.91
Staphylococcus hyicus	270	6.43	0.109	6.38	1.79	2,221	0.190	1,037	3.12
Staphylococcus intermedius	580	7.13	0.074	7.26	1.78	3,279	0.147	1,911	3.53
Staphylococcus lentus	240	6.55	0.108	6.55	1.67	2,206	0.181	1,170	2.81
Staphylococcus saprophyticus	460	6.41	0.083	6.42	1.78	2,158	0.132	1,069	2.84
Staphylococcus sciuri	1,303	6.68	0.048	6.67	1.72	2,506	0.091	1,276	3.29
Staphylococcus simulans	2,047	6.51	0.040	6.44	1.79	2,351	0.069	1,088	3.11
Staphylococcus warneri	1,123	6.44	0.054	6.38	1.81	2,317	0.099	1,043	3.33
Staphylococcus xylosus	1,521	6.41	0.045	6.39	1.77	2,177	0.075	1,048	2.93
Streptococcus canis	192	7.96	0.102	8.10	1.41	4,665	0.296	3,431	4.10
Streptococcus dysgalactiae	6,427	8.00	0.020	8.18	1.63	5,386	0.065	3,625	5.18
Streptococcus gallolyticus	440	8.05	0.071	8.28	1.49	5,064	0.204	3,778	4.28
Streptococcus hyointestinalis	235	8.37	0.091	8.58	1.39	5,882	0.285	4,780	4.37
Streptococcus mitis/oralis	243	8.12	0.096	8.30	1.50	5,530	0.329	3,948	5.13
Streptococcus porcinus	419	7.93	0.075	8.08	1.54	4,896	0.228	3,383	4.67
Streptococcus suis	155	8.04	0.133	8.31	1.66	5,552	0.420	3,960	5.23
Streptococcus thoraltensis	300	7.58	0.094	7.96	1.63	3,873	0.193	3,098	3.35
Streptococcus uberis	18,148	7.90	0.013	8.17	1.70	5,139	0.036	3,594	4.90
Streptococus agalactiae	3,389	7.45	0.030	7.51	1.74	4,108	0.080	2,276	4.66
Trueperella pyogenes	524	8.69	0.062	8.93	1.43	7,420	0.248	6,100	5.67

Table 4 shows the significance of the variables in each group in regard to the overall mean. Group I was characterized by low values for mLS and %ICS and high values of %ISU100. Group III presented high values of mLS and low values of %ISU100. Group IV presented the highest values of mLS and %ICS and lower values for %ISU100. Group II did not show any differences regarding the overall mean. Therefore, considering the results of the other groups, it can be placed between Group I and III.

The maximum α error associated with these groups can also be observed in Fig. 1 and, overall, it matches rather well with the results of the SP analysis.

Discussion

In general, the groups obtained here were quite consistent with previous literature (Djabri *et al.*, 2002; Taponen *et al.*, 2007; Schukken *et al.*, 2009; Kalmus *et al.*, 2011; Supré *et al.*, 2011) and they may provide useful insight about pathogens with low isolation rates regarding other well-known microorganisms. This classification aims to reflect the global trends in the

Table 2. Percentage of isolation in clinical samples (%ICS) and percentage of isolation in samples with SCC under 100,000 cells/mL (%ISU100) for each microorganism identified in in dairy cattle milk samples from Galicia (NW Spain) in relation to the total number of samples in which the microorganism was isolated.

Microorganism	%ICS	%ISU100
Aerococcus viridans	21.48	9.18
Candida famata	34.63	0.31
Candida krusei	42.80	0.28
Candida rugosa	31.74	0.13
Candida tropicalis	39.40	0
Citrobacter koseri	36.41	1.83
Corynebacterium spp.	13.38	13.33
Enterobacter cloacae	34.91	3.86
Enterobacter faecalis	15.39	3.81
Enterococcus faecium	22.32	0.48
Enterococcus saccharolyticus	25.53	0.66
Escherichia coli	62.95	1.37
Klebsiella oxytoca	43.96	4.12
Klebsiella pneumoniae	52.93	0.75
Lactococcus spp.	22.58	2.91

Table 2. Continued.

Microorganism	%ICS	%ISU100
Nocardia spp.	41.90	0.70
Pasteurella multocida	58.27	0
Prototheca spp.	34.10	0.08
Pseudomonas aeruginosa	36.06	2.72
Raoultella ornithinolytica	30.83	7.28
Serratia liquefaciens	23.27	8.93
Serratia marcescens	33.69	1.31
Staphylococcus aureus	20.22	3.30
Staphylococcus chromogenes	20.08	8.87
Staphylococcus epidermidis	12.69	8.25
Staphylococcus gallinarum	15.56	8.50
Staphylococcus haemolyticus	12.02	8.55
Staphylococcus hominis	13.89	11.75
Staphylococcus hyicus	25.74	7.89
Staphylococcus intermedius	18.67	3.98
Staphylococcus lentus	19.02	7.34
Staphylococcus saprophyticus	14.89	9.84
Staphylococcus sciuri	21.37	6.33
Staphylococcus simulans	18.16	6.71
Staphylococcus warneri	17.10	9.49
Staphylococcus xylosus	16.92	9.14
Streptococcus agalactiae	30.40	2.07
Streptococcus canis	29.86	1.06
Streptococcus dysgalactiae	41.48	0.54
Streptococcus gallolyticus	35.71	0.14
Streptococcus hyointestinalis	55.56	0.19
Streptococcus mitis/oralis	39.41	0.73
Streptococcus porcinus	37.61	0.45
Streptococcus suis	37.85	0
Streptococcus thoraltensis	31.54	1.34
Streptococcus uberis	36.45	0.90
Trueperella pyogenes	76.12	0.13

population of the pathogens regarding mLS, %ICS and %ISU100, which is useful to assess the diagnostic significance of an isolation. We expected high mLS and %ICS values in pathogens related to severe mastitis, whilst these values should decrease in pathogens associated to less severe mastitis, presenting high %ISU100 in this case. Pathogens were mainly ordered in that way and, according to PCA, the resulting groups were principally formed based on the values in Dim 1 (Table 3), as it can also be interpreted from the V-test results (Table 4). However, some of them punctually diverged from this order, principally due to the expression of different mLS values from those that could be expected from their %ICS values (Fig. 2b). This shows that some variations with regard to this general tendency are also possible, suggesting a likely more complex epidemiology underlying these results.

Group I contained most of minor pathogens, such as *Corynebacterium* spp. and most of NAS. Pathogens in this group showed the highest %ISU100 and the lowest mLS. Actually, isolations of some species in this group, like *Corynebacterium bovis*, have proved to mostly reflect a colonization of the teat channel rather than a real IMI (Bexiga *et al.*, 2011). However, major pathogens were divided in three groups (II-IV), which seem to indicate certain heterogeneity among them. Group II presented the least virulent one, and it included pathogens like *Staph. aureus* and *Staph. intermedius*, whereas *Strep. agalactiae, Strep. uberis, Strep. dysgalactiae* were classified in Group III. Group IV, the most virulent one, included pathogens like *E. coli* and *Klebsiella pneumoniae*.

Staph. intermedius was the only NAS in the same group as *Staph. aureus* (Group II), suggesting a higher pathogenicity of *Staph. intermedius* in comparison to the rest of NAS. As aforementioned, contradictory findings are reported in NAS mastitis (Jarp, 1991; Taponen *et al.*, 2006; Sampimon *et al.*, 2009; Thorberg *et al.*, 2009; Supré *et al.*, 2011; Fry *et al.*, 2014; Condas *et al.*, 2017). These may reflect differences in NAS isolation frequencies or in the pathogenicity of the strains among the different studied populations, which include, in some cases, a limited number of infections.

Group III included pathogens known to be associated to severe mastitis, but also microorganisms with a discussed role in cattle mastitis, like yeasts or *Prototheca* spp. Yeasts are generally assumed to provoke weak infections rarely associated with clinical mastitis and likely to self-cure (Richard *et al.*, 1980). However, outbreaks with high SCC have also been reported (Malinowsky *et al.*, 2006) and our results seem to be more consistent with this finding. Likewise,

Table 3. Contribution (Contr) and correlation (Corr) of each variable [median of the Linear Score (mLS), percentage of isolation in clinical samples (%ICS) and percentage of isolation in samples with SCC under 100,000 cells/mL (%ISU100)] with each of the three dimensions (Dim 1-3) resulting from the principal components analysis.

Variable	Dimension (% of explained variance for each dimension)					
	Dim 1 (87.46%)		Dim 2 (10.09%)		Dim 3 (2.45%)	
	Contr	Corr	Contr	Corr	Contr	Corr
mLS	36.15%	0.98	1.98%	0.08	61.8%	0.21
%ICS	30.84%	0.90	61.05%	0.43	8.11%	-0.08
%ISU100	33.01%	-0.93	36.97%	0.33	30.02%	0.15

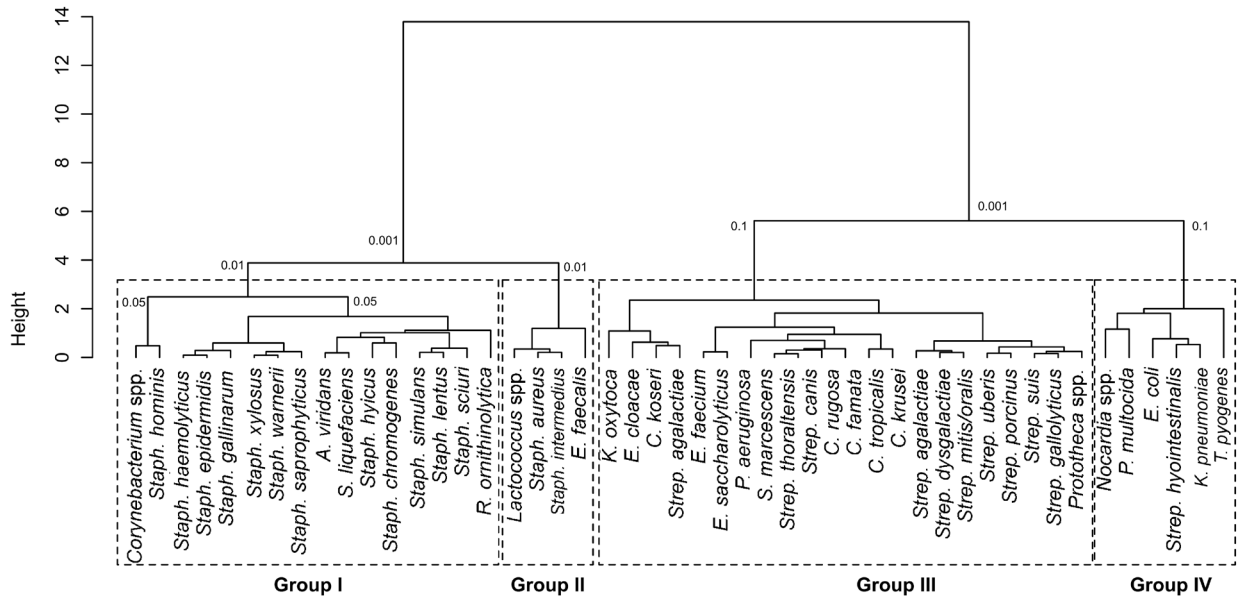

Figure 1. Dendrogram with the groups of pathogens resulting from the cluster analysis. Numbers indicate the maximum α error for each branch.

mastitis caused by *Prototheca* spp. is also considered to be rarely associated with clinical signs (Scaccabarozzi *et al.*, 2008), but we also found this genus in Group III. This could indicate that these kinds of infections are responsible for mastitis of relative importance. It is also noteworthy that *Pseudomonas aeruginosa*, described as a cause of chronic mastitis which can also provoke either acute or subacute processes or subclinical disease (Hogan *et al.*, 1989), was classified in Group III in this study along with other pathogens responsible of severe mastitis.

Group IV was formed by mammary pathogens rarely isolated in mastitis samples such as *T. pyogenes* and *Nocardia* spp. This was not an unexpected finding since both pathogens had already been associated with severe mastitis (Packer, 1977; Tarabla *et al.*, 1993). However, according to our results, *Pasteurella multocida* and *Strep. hyointestinalis* could also be associated with processes of similar severity.

It is interesting that despite pathogens with %ISU100 >5% were included in Group I, several pathogens included in Groups II and III presented %ISU100 over 2%. Ranges and means of SCC in IMI vary among different pathogens (Djabri *et al.*, 2002), so their isolation frequency is also expected to vary in samples with low SCC. The diagnostic importance of isolations in samples with low SCC may vary depending on the pathogen. Thus, pathogens of Group I in samples with low SCC may indicate mild disease or colonization without IMI, but major pathogens isolated in this type of samples may be interpreted as an early indication of IMI (Schwarz *et al.*, 2010) and their detection would be of importance in cattle mastitis programs. Moreover, higher %ISU100 were found in pathogens usually transmitted from other infected animals (e.g., *Staph. aureus* and *Strep. agalactiae*) than in pathogens with an environmental origin (e.g., *E. coli* or *Strep. uberis*) (Table 2). This fact has a great importance for control, thus calling for the implementation of new biological markers in order to get a more accurate information of the actual udder health status of dairy cows (Damm *et al.*, 2017).

It is also noteworthy that pathogens traditionally merged in microbiological groups were separated in different clusters in our study, showing potential variability in their pathogenic role (Fig. 1). This could explain some of the divergences found in the literature for species of the genera *Enterococcus, Aerococcus* or *Lactococcus*. Species within these genera still have a discussed role in cattle mastitis (Devriese *et al.*, 1999; Zadoks *et al.*, 2011) and while some authors consider them to be minor pathogens (Guélat-Brechbuehl *et al.*, 2010), outbreaks of clinical mastitis involving some of them have also been reported (Todhunter *et al.*, 1995). This kind of differences had already been described for some pathogens among the *Enterobacteriaceae* family. For example, *E. coli* and *Klebsiella* spp. had been attributed to the most severe cases, whereas less clinical severity had been observed in infections caused by *Serratia* spp. and *Enterobacter* spp., which were more frequently related to chronic mastitis (Schukken *et al.*, 2012). Since frequencies of isolation of the species

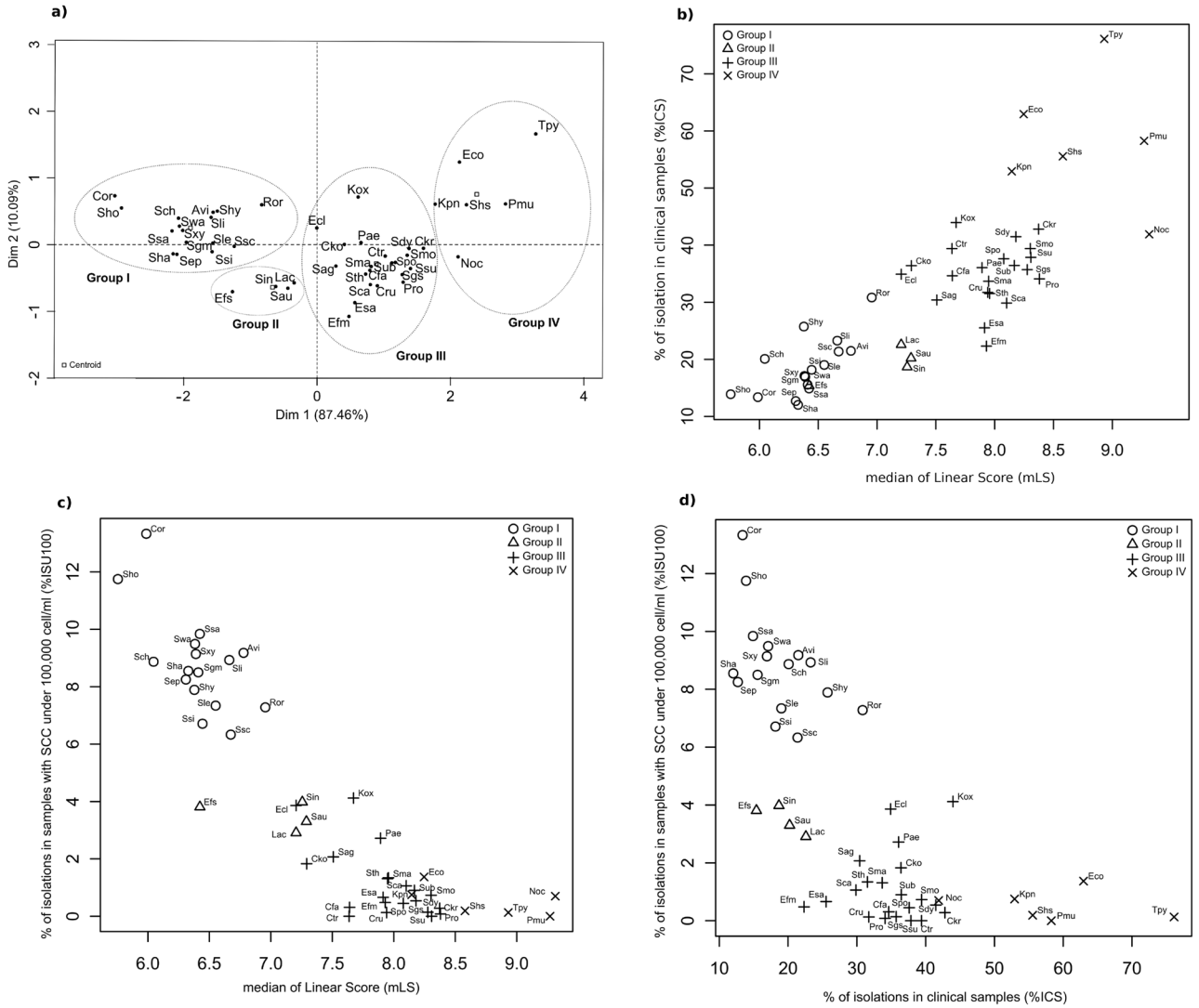

Figure 2. Individual factor map of the principal component analysis (a) and pairwise plots for median of lineal score (mLS), % of isolation in clinical samples and % of isolations in samples with SCC under 100,000 cell/mL values of each pathogen isolated in in dairy cattle milk samples from Galicia (NW Spain) (b, c, d). Avi: *Aerococcus viridans*, Cfa: *Candida famata*, Ckr: *Candida krusei*, Cru: *Candida rugosa*, Ctr: *Candida tropicalis*, Cko: *Citrobacter koseri*, Cor: *Corynebacterium* spp., Efs: *Enterococcus faecalis*, Efm: *Enterococcus faecium*, Esa: *Enterococcus saccharolyticus*, Ecl: *Enterobacter cloacae*, Eco: *Escherichia coli*, Kos: *Klebsiella oxytoca*, Kpn: *Klebsiella pneumoniae*, Lac: *Lactococcus* spp., Noc: *Nocardia* spp., Pmu: *Pasteurella multocida*, Pro: *Prototheca* spp., Pae: *Pseudomonas aeruginosa*, Ror: *Raoultella ornithinolytica*, Sma: *Serratia marcescens*, Sli: *Serratia liquefaciens*, Shy: *Staphylococcus hycus*, Sau: *Staphylococcus aureus*, Sch: *Staphylococcus chromogenes*, Sep: *Staphylococcus epidermidis*, Sgm: *Staphylococcus gallinarum*, Sha: *Staphylococcus haemolyticus*, Sho: *Staphylococcus hominis*, Sin: *Staphylococcus intermedius,* Sle: *Staphylococcus lentus*, Ssa: *Staphylococcus saprophyticus*, Ssc: *Staphylococcus sciuri*, Ssi: *Staphylococcus simulans*, Swa: *Staphylococcus warneri*, Sxy: *Staphylococcus xylosus*, Sag: *Streptococcus agalactiae*, Sca: *Streptococcus canis*, Sdy: *Streptococcus dysgalactiae,* Shs: *Streptococcus hyointestinalis*, Smo: *Staphylococcus mitis/oralis*, Spo: *Streptococcus porcinus*, Ssu: *Streptococcus suis*, Sth: *Streptococcus thoraltensis*, Sub: *Streptococcus uberis*, Sgs: *Streptococcus gallolyticus*, Tpy: *Trueperella pyogenes*.

that compose these groups may vary among studies and these pathogens may be implicated in mastitis of different severity, our results suggest that merging these species may lead to incorrect inferences of their role in cattle mastitis.

It has to be noted that it is impossible to evaluate all the factors related to the severity of mastitis in this kind of study (such as the duration of the infection or the severity of the exhibited signs in clinical mastitis). Cattle mastitis is dependent on different factors that

Table 4. Results of the V-test and means of the median of linear score (mLS), percentage of isolations in clinical samples (%ICS) and percentage of isolations in samples with somatic cell counts under 100,000 cells/mL (%ISU100) for each group resulting from cluster analysis.

Variable	Mean	SEM	Group I[1]		Group II[2]		Group III[3]		Group IV[4]	
			V-test	mean	V-test	mean	V-test	mean	V-test	mean
mLS	7.44	0.13	-5.58	6.48	NS	7.10	3.32	7.89	3.73	8.70
%ICS	31.00	2.08	-4.30	19.18	NS	19.21	Ns	35.31	4.95	55.26
%ISU100	3.87	0.57	6.27	8.36	NS	3.5	-4.36	1.43	-2.24	0.62

[1]Group I: *Corynebacterium* spp., *Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus epidermidis, Staphlylococcus gallinarum, Staphylococcus xylosus, Staphylococcus warnerii, Staphylococcus saprophyticus, Aerococcus viridans, Serratia liquefaciens, Staphylococcus hyicus, Staphylococcus chromogenes, Staphylococcus simulans, Staphylococcus lentus, Staphylococcus sciuri, Raoultella ornithinolytica*. [2]Group II: *Lactococcus* spp., *Staphylococcus aureus, Staphylococcus intermedius, Enterococcus faecalis*. [3]Group III: *Klebsiella oxytoca, Enterobacter cloacae, Citrobacter koseri, Streptococcus agalactiae, Enterococcus faecium, Enterococcus saccharolyticus, Pseudomonas aeruginosa, Serratia marcescens, Streptococcus thoraltensis, Streptococcus canis, Candida rugosa, Candida famata, Candida tropicalis, Candida krusei, Streptococcus dysgalactiae, Streptococcus mitis/oralis, Streptococcus uberis, Streptococcus porcinus, Streptococcus suis, Streptococcus gallolyticus, Prototheca* spp. [4]Group IV: *Nocardia* spp., *Pasteurella multocida, Escherichia coli, Streptococcus hyointestinalis, Klebsiella pneumoniae, Trueperella pyogenes.* NS: non-significant in this group.

may affect to the presentation and severity of clinical signs and SCC. Differences in pathogenicity have been observed among strains in both SCC (Zadoks *et al.*, 2002) and clinical outcomes (Middleton & Fox, 2002). In addition, there are also cow-specific characteristics that may lead to wide variations in the course of IMI such as parity, month of lactation or SCC in previous lactation (Steeneweld *et al.*, 2008; Oliveira *et al.*, 2015). Including cow and pathogen factors in the cluster analysis would have provide a more robust result and it would be necessary to a more complete and accurate determination of the pathogenicity of these pathogens, but unfortunately, we could not register animal or herd level information in this study and so, a certain degree of bias is expected. However, since we have analyzed a large number of isolations by pathogen we aimed to be representative of the global trends of each pathogen in a population. Nevertheless, it must be noted that a different cluster solution may be expected in other populations due to differences of cow-specific factors or in the circulating strains. Despite that, our classification is pretty consistent with previous literature (Djabri *et al.*, 2002) and it allows the relative comparison among pathogens. This is particularly interesting for microorganisms with little isolations since their available epidemiological information is limited and it is frequently derived from few herds, which may be influenced by particular characteristics of the animals and herds analyzed. An overrepresentation of severe mastitis in samples submitted for diagnosis is also expected in comparison to a random sampling, so it is possible a trend to over-associate microorganisms to more severe cases. Actually, and probably as a consequence of the design of our study, our SCC values were higher than those reported by Djabri *et al.* (2002) for the pathogens they included in a meta-analysis to evaluate the associated effects of bacteria or group of bacteria with IMI. However, in our opinion, this does not affect our aims, since this study does not intend to establish concrete values for each pathogen, but to perform a relative comparison between the analyzed pathogens.

This study provides a picture of some of the outcomes related to the pathogenicity of several pathogens involved in cattle mastitis. Our results are also helpful for the understanding of the importance and frequency of isolation of the different pathogens in samples with low SCC. The groups obtained in this study can aid, together with other factors, in the classification of mammary pathogens. The classification of microorganisms is more complex than the traditional differentiation between minor and major pathogens, especially in the latter case. Our results provide a starting point for the interpretation of the information of pathogens with low prevalence, and highlight the differences among pathogens which are frequently merged in microbiological groups for their study. Further studies, in which other epidemiologic characteristics are added, could aid in understanding the differences in the epidemiology of mastitis pathogens.

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