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Companilactobacillus alimentarius: An extensive characterization of strains isolated from spontaneous fermented sausages

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ABSTRACT

Companilactobacillus alimentarius is a facultatively heterofermentative lactic acid bacterium (LAB) that is a significant constituent within the microbiota of various traditional fermented foods exerting several functions in fermentative or ripening processes. This species has been isolated from Spanish fermented sausages, where its frequency of isolation was comparable to those of *Latilactobacillus sakei* and *Latilactobacillus curvatus*. Despite to its presence in several niches, ecological information on this species is still scarce and only few publications report information about its safety features (i.e. antibiotic resistance). Since studies on *C. alimentarius* concern the analysis of a few individual traits regarding this species, a more extensive work on a larger number of isolates from the same matrix have been performed to allow a clearer interpretation of their phenotypic and technological characteristics. Specifically, 14 strains of *C. alimentarius* isolated from Mediterranean spontaneously fermented sausages, have been screened for their safety and technological characteristics (such as antibiotic resistance, biogenic amine production, inhibiting potential, growth at different temperatures and NaCl concentrations) and with phenotype microarrays with the aim to elucidate their potential role and contribution to sausage fermentation and ripening.

In general, a wide variability was observed in relation to the parameters considered. Several of the tested strains were able to produce histamine, tyramine and putrescine while the antibiotic resistance greatly varied according to the strains, with the exception of vancomycin. In addition, *C. alimentarius* strains showed a relevant potential to grow in conditions of salt and temperature mimicking those found in fermented foods. In particular, the growth at 10 °C and in the presence of salt can explain the presence of *C. alimentarius* in sausages and its adaptation to fermented meat environment in which low temperature can be applied during ripening. The differentiation of the phenotypic profile reflected the environmental conditions that influenced the isolation source, including those derived by the raw materials.

Given the species frequent association with spontaneous fermentations or the ripening microbiota of various products, despite not being intentionally used as starter cultures, the data presented in this study contribute to a deeper comprehension of their role, both advantageous and detrimental, in numerous significant fermented foods.

1. Introduction

Companilactobacillus (formerly *Lactobacillus*) *alimentarius* is a Gram positive facultatively heterofermentative lactic acid bacterium (LAB) for which ecological information is still scarce (Zheng et al., 2020). Even if members of this species are not commonly used as starter cultures, they are significant constituents within the microbiota of various traditional fermented foods and fulfil crucial functions in fermentative or ripening

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processes (Bassi et al., 2022; Fujimoto et al., 2019; García Fontán et al., 2007a, 2007b). Their optimal temperature ranges between 25 and 30 °C and they can grow at 15 °C and at 37 °C using pentoses, hexoses and disaccharides as carbon sources; the genome size is 2.34 Mbp and the mol% G + C content of DNA is 35.4. (Zheng et al., 2020). In addition, the species *C. alimentarius* is recognized under the Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) (EFSA, 2020) and is part of the inventory of microbial food cultures with safety demonstration in fermented food products (Bourdichon et al., 2022).

Despite the relative scarcity of information on its physiological role, the presence of *C. alimentarius* is particularly relevant in fermented foods obtained from vegetable matrices (Guo et al., 2022; Liang et al., 2022). This species has been found in wheat (Randazzo et al., 2005), rye (Corsetti et al., 2001) and whole soft wheat sourdoughs (Taccari et al., 2016), although not as a dominant population. According to Fujimoto et al. (2019), *C. alimentarius* was the second species, after *Levilactobacillus brevis*, isolated from Japanese sourdough. It is also present in some traditional fermented products, such as Tarhana, obtained from wheat flour, yogurt, vegetables, and spices (Özel et al., 2020). Other spontaneously fermented vegetables are characterized by the presence of this species. For example, during the fermentation of pickled chayote (*Sechium edule*), *C. alimentarius* was the prevalent species, accompanied by other LAB and yeasts (Shang et al., 2022), as well as in other Chinese fermented foods obtained from *Brassica juncea*, *Raphanus sativus* and *Capsicum annuum* (Liu and Tong, 2017).

In addition to vegetable matrices, this species has been often isolated from fermented meats. In their survey on the microbial communities of European fermented sausages, Van Reckem et al. (2019) found only sporadic presence of *C. alimentarius*. Nevertheless, strains belonging to this species were isolated from Spanish fermented sausages, such as Androlla (García Fontán et al., 2007a) and Botillo (García Fontán et al., 2007b), where their frequency of isolation was comparable to those of *Latilactobacillus sakei* and *Latilactobacillus curvatus*. The relevant presence of *C. alimentarius* was observed also in Turkish fermented sausages (sucuk) by Gürakan et al. (1995) and Kesmen et al. (2012). A potential probiotic strain of *C. alimentarius* has also been used as starter cultures for the production of fermented Scandinavian sausages by Klingberg et al. (2005).

C. alimentarius has also been associated with dairy products. An interesting work of Cardinali et al. (2017) demonstrated that this bacterium was present in the phyllosphere of *Carlina acanthifolia*, a plant traditionally used for vegetable rennet production, and is able to pass through the rennet, in the goat milk, affecting the early bacterial dynamics during cheesemaking. This species has been also tested as probiotic for promoting goat intestinal health and producing milk with higher concentrations of unsaturated fatty acid (Apás et al., 2015).

From a bioprotective perspective, certain strains of *C. alimentarius* have exhibited the capability to produce bacteriocins, which have been subjected to preliminary investigations to assess their efficacy against *Bacillus* spp., the causative agents of bread rope (Menteş et al., 2007). Hu et al. (2017) demonstrated the production of a bacteriocin (lactocin MM4) with a broad inhibitory range towards several Gram-positive and Gram-negative bacteria as well as against some fungi. In addition, it was demonstrated that members of this species can produce phenyllactic and 4-hydroxy-phenyllactic acids, metabolites active against spoiling moulds (Valerio et al., 2004). The bioprotective effect of the strain Floracarn L2 against *Listeria monocytogenes* has been described in meat (Juven et al., 1998). Another study demonstrated the capacity of this species together with *Staphylococcus xylosus* to act as protective cultures in under vacuum sliced cooked ham (Kotzekidou and Bloukas, 1998). On the other hand, in fishery products *C. alimentarius* was responsible for herring spoilage, causing bulging of lids and gas formation (Lyhs et al., 2001).

Concerning safety aspects, few publications report information on the antibiotic resistance profile of this species. According to Gevers et al. (2003), two strains of this species harbour a plasmid-located *tet(M)* gene with transfer capacity to *Enterococcus faecalis*, while in a study of Campedelli et al. (2019) members of this species were found to display *tet*(S) gene and *Isa* gene encoding for resistance to clindamycin. Despite its diffusion in several spontaneous fermented foods, few information is available concerning its ability to produce biogenic amines (BAs). Previous studies reported that one strain isolated from fermented food, was able to produce tyramine (Straub et al., 1995), while another study showed no decarboxylase activity in a strain isolated from Himalayan fermented foods (Dewan and Tamang, 2007), as well as in a strain from table olives (Yalçınkaya and Başyiğit Kılıç, 2019). Recently, a metagenomics study on the microbial communities present in Mediterranean spontaneously fermented sausages showed that *Companilactobacillus* spp. was among the prevalent genera in salamis, some of them characterized by the presence of relevant amounts of BAs (Barbieri et al., 2021).

To date, studies on *C. alimentarius* concern the analysis of a few individual traits regarding this species, but there is a lack of more extensive works on a larger number of isolates from the same matrix allowing a clearer interpretation of their phenotypic and technological characteristics. The aim of this work was to perform a wider characterization of 14 strains of *C. alimentarius* isolated from Mediterranean fermented sausages. In order to elucidate the potential role and contribution of *C. alimentarius* strains in sausage fermentation and ripening, all strains were firstly screened for phenotype microarrays and then characterized for aspects concerning safety and technological issues (such as antibiotic resistance, BAs production, inhibiting potential, growth at different temperatures and NaCl concentrations).

2. Materials and methods

2.1. *Companilactobacillus alimentarius* microbial strains

The strains used in this study were isolated from Spanish spontaneously fermented sausages (Andalusia region), in which the presence of *Companilactobacillus* spp. was detected through metagenomic analysis (Barbieri et al., 2021) and strains belonging to the species *C. alimentarius* were isolated from the ripened products (Bassi et al., 2022). The list of the 14 strains considered in this study is reported in Table 1.

Pure cultures were stored at −20 °C in De Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) containing 20 % glycerol (Sigma Aldrich) until further analyses.

2.2. *Antimicrobial activity against food-borne pathogens*

The antimicrobial activity of the 14 *C. alimentarius* strains was evaluated through an agar spot test against *L. monocytogenes* Scott A and

Table 1
Source of isolation of the 14 strains considered in this study, relative abundance (%) of *Companilactobacillus* spp. detected by metagenomic analysis and percentage of isolation of *C. alimentarius* on all the isolated strains (adapted from Barbieri et al. (2021) and Bassi et al. (2022)).

Source of isolation	Relative abundance (%) of *Companilactobacillus* detected by metagenomic analysis	% of isolation of *C. alimentarius* on all the isolated strains	*C. alimentarius* strains studied
Chorizo Bérchules	45.0	87.5	CB1, CB6, CB8, CB16, CB22, CB31, CB36, CB41, CB43
Chorizo Écija	34.5	4.5	CE49
Chorizo Olvera	5.3	57.0	CO12, CO24, CO50
Salchichón Écija	55.3	12.5	SE14

Salmonella enterica serovar Enteritidis 155, belonging to the collection of the Department of Agricultural and Food Science of the University of Bologna.

The foodborne pathogens were grown into Brain Heart Infusion (BHI) medium (Oxoid), while LAB strains into MRS agar (Oxoid). Each culture was incubated overnight at 30 °C. Target pathogens were inoculated in BHI soft agar (0.7 %) plates to obtain a final concentration of 6 log CFU/ml to form a bacterial lawn. Once the plate was dried, 10 μl drop of each *C. alimentarius* strain cultures were spotted onto plates. The samples were observed after 24 h of incubation at 30 °C and the absence/presence of inhibition zones was evaluated. The inhibitory activity was expressed based on the diameter halo around the spot: + (≤0.5 cm), ++ (0.5–2 cm), +++ (>2 cm), or - (no halo). Cell free supernatants (CFS) of *C. alimentarius* strains that showed an antimicrobial activity were collected after centrifugation at 6000 rpm for 10 min and filtration with polyethersulfone (PES) membrane (Merck Millipore, Carrigtwohill, Ireland) with a pore size of 0.22 μm. The CFS were collected in sterile microcentrifuge tubes and tested, both unmodified and neutralised at pH 6.5 with NaOH 1 M, through the well technique against the same pathogens. Once the indicator strain has grown, the appearance of inhibition halos around the wells was observed and measured in millimeters to detect the inhibitory activity. Three independent tests were carried out, and each sample was tested in duplicate.

2.3. Phenotype microarray of C. alimentarius strains

The screening of *C. alimentarius* strains was performed with a phenotype microarray (OmniLog®, Biolog, Inc., Hayward, USA), using Gen III MicroPlate, according to the manufacturer's instructions. For each strain, colonies cultured onto MRS agar medium were resuspended into inoculating fluid C (Biolog, Inc.), until reaching a microbial cell concentration from 90 % to 98 % T (the light transmittance measured by OmniLog® turbidimeter). Briefly, 100 μl of each cell suspension were inoculated into the MicroPlate wells and incubated at 30 °C for 96 h in accordance with growth characteristics. Measurement of strain metabolism was assessed by colorimetric redox assay and all MicroPlates were read every 15 min. The data were collected with the OmniLog® and companion computer software.

2.4. Antibiotic-resistance profile

Antibiotic-resistance profile of the strains was assessed considering EFSA indications (EFSA, 2012). A minimum inhibitory concentration (MIC) test to evaluate the resistance to ampicillin (Amp), chloramphenicol (Chl), clindamycin (Cli), erythromycin (Ery), gentamicin (Gen), tetracycline (Tet), kanamycin (Kan) and streptomycin (Str) was performed using micro dilution technique in the recommended Lymphocyte Separation Medium (LSM) medium (Iso-Sensitest™ broth 90 % and MRS broth 10 %; ThermoFisher Scientific) (ISO, 2010). Results were collected after 48 h of incubation at 30 °C and the presence of resistance for each antibiotic is defined according to the cut off reported by EFSA: Gen = 16, Kan = 64, Str = 64, Tet = 8, Ery = 1, Clin = 1, Chlor = 4, Amp = 4 (EFSA, 2012).

2.5. Biogenic amines production

The amino biogenic potential of *C. alimentarius* strains was tested through the screening in Bover-Cid-Holzapfel medium (BC) (Bover-Cid and Holzapfel, 1999). All strains were pre-cultivated in MRS broth and then inoculated in BC broth, supplemented with the biogenic amine (BA) precursors (histidine, tyrosine, ornithine or lysine) and incubated at 30 °C for 72 h. The supernatants of presumptive positive strains were collected and stored at −20 °C, until the HPLC analysis. After a dansyl-chloride derivatization (Sigma-Aldrich, St Louis, USA), samples were injected into HPLC Agilent Technologies 1260 Infinity with the automatic injector (G1329B ALS 1260, loop of 20 μl), equipped with a C18 Waters Spherisorb ODS-2 (150 × 4.6 mm, 3 μm) column and a UV detector (G1314F VWD 1260) set at 254 nm, to confirm the BA production (histidine, tyramine, putrescine and cadaverine) according to the method reported by Montanari et al. (2023). The BA amount was measured with reference to a calibration curve obtained through the injection of dansyl-chloride-derivatized BA standards. Under the adopted conditions, the detection limit for all compounds was 3 mg/l. All the analyses were performed in triplicate.

2.6. Growth performances in presence of different salt concentrations and at different incubation temperatures

The growth performances of *C. alimentarius* strains were evaluated in relation to different salt concentrations (0 %, 2.5 % and 5 % NaCl) at 20 °C and at different incubation temperatures (10 °C, 20 °C and 30 °C). They were pre-cultivated in MRS broth for 24 h at 30 °C and then inoculated at a final concentration of 5 log CFU/ml in the different media chosen for the analyses. Their growth was monitored through the variation of optical density at 600 nm (OD_{600}) with time (t), measured with an UV-VIS spectrophotometer 6705 UV–Vis (Jenway, Stone, UK). The collected data were elaborated with Gompertz Eq. (1), as modified by Zwietering et al. (1990):

$$OD_{600} = A \cdot e^{-e^{\left(\left(\frac{\mu_{max} \cdot e}{A}\right) \cdot (\lambda - t) + 1\right)}} \quad (1)$$

where A represent the maximum OD_{600} value reached, μ_{max} is the maximum OD_{600} increase rate (OD_{600} h^{-1}) and λ is the lag phase (h).

Moreover, pH values were also monitored overtime by pH-meter Basic 20 (Crison Instruments). The data were modelled with the same equation modified as follow (Eq. (2)):

$$pH = k + A_{pH} \cdot e^{-e^{\left(\left(\frac{\mu_{pHmax} \cdot e}{A_{pH}}\right) \cdot \left(\lambda_{pH} - t\right) + 1\right)}} \quad (2)$$

where k is the higher asymptote of the curve (initial pH), A_{pH} is the lower asymptote of the curve (final pH decrease), μ_{pHmax} is the maximum pH decrease rate (pH h^{-1}) and λ_{pH} is the lag phase (h).

2.7. Statistical analysis

The parameters of the OD_{600} and pH curves were estimated using Statistica 8.0 software (StatSoft Inc., Tulsa, USA). The distribution of the modelled parameters was tested with ANOVA to define statistically significant differences. Statistical differences were considered significant at a level of $p \leq 0.05$ using the Tukey test. ANOVA and Box and Whiskers plots were obtained by using the statistical software R (R Core Team, 2020).

3. Results and discussion

3.1. Antimicrobial activity of the C. alimentarius strains against Salmonella and Listeria

The 14 strains of *C. alimentarius* were tested for their capability to counteract the growth of *L. monocytogenes* ScottA and *S.* Enteritidis 155.

The cell suspensions of all 14 strains showed a similar inhibition against the target pathogens, being in general slightly more active against *S.* Eenteritidis 155 rather than *L. monocytogenes* (0.5–2 cm halo diameter versus ≤0.5 cm). The same observations can be done for CFS (data not shown), but this activity was not present after pH neutralization, indicating that the bioactivity was only due to acidification and no specific bacteriocins against the target pathogens were produced.

Table 2
Companilactobacillus alimentarius behaviour in relation to carbon sources and limiting growth conditions in GEN III MicroPlates: + positive, ± weak, − negative.

Strains	Carbon sources												Chemical sensitivity																							
	D-Maltose	D-Trehalose	D-Cellobiose	Sucrose	α-D-Lactose	β-Methyl-D-Glucoside	D-Salicin	N-Acetyl-D-Glucosamine	α-D-Glucose	D-Mannose	D-Fructose	D-Galactose	pH 6	pH 5	1% NaCl	4% NaCl	8% NaCl	1% Sodium Lactate	Fusidic Acid	D-Serine	Troleandomycin	Rifamycin SV	Minocycline	Lincomycin	Guanidine HCl	Niaproof 4	Vancomycin	Tetrazolium Violet	Tetrazolium Blue	Nalidixic Acid	Lithium Chloride	Potassium Tellurite	Aztreonam	Sodium Butyrate	Sodium Bromate
CB1	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	-	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	-
CB6	+	+	-	-	+	-	-	+	+	+	+	±	+	+	+	+	-	+	±	+	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+
CB8	-	-	-	-	-	±	-	+	+	+	+	-	+	+	+	±	-	+	+	±	-	-	+	-	-	-	+	+	+	+	-	+	+	+	-
CB16	-	-	-	±	-	-	-	+	+	+	+	-	+	+	+	±	-	+	+	-	-	-	+	-	-	-	+	+	+	+	-	+	+	+	-
CB22	-	-	-	+	-	±	+	+	+	+	±	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+
CB31	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	±	-	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	+	+	-
CB36	-	-	-	-	-	-	-	+	+	-	-	-	+	+	+	±	-	+	+	-	-	-	+	-	-	-	+	+	+	±	-	+	-	+	±
CB41	-	-	-	-	-	±	-	+	+	-	+	-	+	+	+	±	-	+	+	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	-
CB43	-	-	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-	±	-	-	-	-	+	+	+	+	-	+	+	+	-
CE49	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	-	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	-
CO12	-	-	-	+	-	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	+	+	+	-
CO24	-	-	-	-	-	+	±	±	+	+	+	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	+	+	+	-
CO50	-	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+
SE14	-	+	+	±	-	+	+	+	+	+	+	-	+	+	+	±	-	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	-

3.2. Screening using phenotype microarray

In Table 2, the results of the GenIII MicroPlate test concerning carbon sources and chemical sensitivity are reported. Fifty-nine tests resulted negative for all the strains (data not shown). Concerning the carbon sources, 12 of them were used by at least one strain. In particular, all strains grew on α-D-glucose and the majority was able to use D-mannose (with the exception of CB36, CB41 and CE49), D-fructose (except CB36 and CE49) and *N*-acetyl-D-glucosamine (except CB31 and CE49). D-maltose and α-D-lactose were fermented only by CB6, which metabolised also D-trehalose (with SE14) and showed a weak growth on D-galactose. The strain SE14 was the only able to grow on D-cellobiose, while sucrose was fermented by CB22, CB43, CO12 and, at a lesser extent, by SE14 and CB16. The strains CB43, CB22, CO24, CO50 and SE14 used D-salicin and β-methyl-D-glucoside as carbon sources. According to Randazzo et al. (2005), the four strains of *C. alimentarius* isolated from sourdoughs were all able to ferment glucose, fructose, maltose and sucrose, while according to Hu et al. (2017) the strain FM-MM$_4$ fermented glucose, fructose, galactose, trehalose and lactose. García Fontán et al. (2007b) found that almost all the strains (96 %) of this species isolated from a Spanish fermented sausage (*Botillo*) can ferment sucrose, but none of them fermented lactose.

The growth in the presence of chemical sensitivity showed that all the strains were able to grow at pH 6 and 5 and when NaCl was added at 1 and 4 %, as well as with sodium lactate at 1 %. Only the strain CO50 grew also at 8 % NaCl. Concerning antibiotics, all the strains grew in the presence of vancomycin, nalidixic acid and fusidic acid, while four of the strains could grow with minocycline (CB8, CB16, CB31 and CB36). All the strains gave a positive response in the presence of tetrazolium violet, tetrazolium blue, potassium tellurite and sodium butyrate, but none with lithium chloride.

3.3. Antibiotic resistance

The 14 strains of *C. alimentarius* were tested for their antibiotic resistance by considering the 8 antibiotics indicated by EFSA (2012) for facultative heterofermentative lactobacilli. The results are reported in Table 3.

The strain CB8 was the only one resistant to Kan, Tet and Ery. The same strain was also resistant to Chl, together with CB43 and CO12. Two strains were resistant to Gen (CO12 and SE14, 14.2 % of the total), three to clindamycin (CB41, CO12 and CO24, 21.3 %) and four to Str (CE49, CO12, CO50 and SE14, 28.4 %), while all the strains were sensitive to Amp. Overall, 6 strains were sensitive to all antibiotics considered and five were resistant to one antibiotic. The strains SE14 was resistant to two antibiotics while the strains CB8 and CO12 were resistant to four of the eight antibiotics tested.

In general, also for LAB species recognized as QPS (including *C. alimentarius*) and involved in food fermentations, antibiotic resistance should be assessed (Campedelli et al., 2019; Colautti et al., 2022; Klingberg et al., 2005). However, few studies are available concerning this aspect in *C. alimentarius* (Gevers et al., 2003).

Fermented meats can be a reservoir of antibiotic resistant LAB strains and the genes responsible for resistance can be horizontally transmissible to other species, including pathogens (Belloso Daza et al., 2022). Due to these factors, recent reports from EFSA suggest the need for further investigations on LAB involved in food fermentations (EFSA, 2012, 2021).

The resistance against aminoglycosides like gentamicin, streptomycin and kanamycin has a chromosomal origin in many LAB, even if its transmissibility through mobile genetic elements has been described in lactobacilli and enterococci (Werner, 2012; Zarzecka et al., 2022; Rozman et al., 2023). However, according to the data obtained in the present work, the resistance to this class of antibiotics did not seem to be a species characteristic and could be linked to genetic mobile elements. According to Campedelli et al. (2019), the resistance of type-strains assigned to the genus *Companilactibacillus* (formerly *Lactobacillus alimentarius* group) was approx. 20 % for gentamicin, 45 % for streptomycin and 80 % for kanamycin, while in this case, a percentage of 14.3 %, 28.6 % and 7.1 % was observed, respectively, for the same antibiotics. Concerning tetracycline, Gevers et al. (2003) found a plasmid located *tetM* gene in a strain of *C. alimentarius* isolated from fermented

Table 3
Antibiotic-resistance profile of *Companilactobacillus alimentarius* strains, assessed following EFSA indications (Gen = Gentamicin; Kan = Kanamycin; Str = Streptomycin; Tet = Tetracycline; Ery = Erythromycin; Clin = Clindamycin; Chlor = Chloramphenicol; Amp = Ampicillin). The MIC value are reported and the presence of resistance for each antibiotic is highlighted in bold (cut off: Gen = 16, Kan = 64, Str = 64, Tet = 8, Ery = 1, Clin = 1, Chlor = 4, Amp = 4).

Strains	Gen	Kan	Str	Tet	Ery	Clin	Chlor	Amp
CB1	16	64	64	8	0.125	1	2	4
CB6	2	2	16	1	0.125	0.032	2	0.5
CB8	4	**128**	16	**16**	**4**	0.125	**8**	2
CB16	8	16	32	4	0.5	0.125	4	1
CB22	4	16	32	2	0.5	0.5	4	1
CB31	8	4	16	4	0.25	0.063	4	1
CB36	8	16	16	2	0.25	0.032	4	1
CB41	4	16	16	2	0.5	**4**	2	1
CB43	2	16	64	4	1	1	**8**	2
CE49	16	64	**128**	1	0.25	1	4	2
CO12	**32**	64	**128**	2	1	**4**	**8**	4
CO24	4	16	32	2	0.25	**4**	2	2
CO50	4	16	**256**	2	0.25	0.125	2	1
SE14	**32**	64	**256**	2	1	1	4	2

sausages and demonstrated the possibility to transfer this plasmid to a strain of *Ent. faecalis*. The same gene was found in several strains belonging to the species *Lat. sakei*, *Lat. curvatus*, *Lactiplantibacillus paraplantarum*, *Lcb. paracasei* from meat fermentations (Bassi et al., 2022; Fraqueza, 2015; Zonenschain et al., 2009). Also in this case, the incidence of resistant strains (7.1 %) was lower than that observed by Campedelli et al. (2019) for species of the genus *Companilactibacillus*, which were characterized by approx. 40 % of resistance. Also, for the macrolide erythromycin, plasmid associate genes (*erm*, *msr* and *mef* genes) responsible for acquired resistance has been found in lactobacilli (Leclercq and Courvalin, 1991; Zonenschain et al., 2009; Comunian et al., 2010) and the possibility to transfer this plasmid to other LAB (*Ent. faecalis*) has been demonstrated (Nawaz et al., 2011). However, no information concerning specifically *Companilactobacillus* is available in literature. In this study, the percentage of clindamycin resistant strains (21.4 %) confirmed the results reported for Italian sausages by Federici et al. (2014), while the resistance to chloramphenicol (21.4 %) was higher than those reported in other similar studies (Aymerich et al., 2006).

Since this investigation cannot clarify the transmissibility or the type of mechanisms responsible for the resistance observed, further studies are needed to understand if these bacteria, present as component of the ripening microbiome of sausages and many other fermented products, can be responsible for the diffusion of antimicrobial resistance (AMR) genes to other microbial species.

3.4. Biogenic amine production

The strains of *C. alimentarius* were isolated from sausages in which tyramine (from 67 to 202 mg/kg), putrescine (from 79 to 156 mg/kg) and, as far as one sample, histamine (174 mg/kg) were detected (Barbieri et al., 2021; Bassi et al., 2022). For this reason, the strains were tested for their ability to produce BA in BC medium. The amounts of each BA detected after 72 h of incubation in BC medium is reported in Table 4.

Nine strains out of 14 (64.3 %) produced tyramine, even if at different extent. Four strains (CB1, CB6, CO12 and CO24) produced low amounts of this aromatic BA, ranging from 61.5 to 15.1 mg/l, while the other strains accumulated high level of tyramine. In fact, the strains CB8, CB16 and CB41 produced concentration of tyramine ranging from 386.1 to 489.2 mg/l, while extremely high concentrations were produced by the strains CE49 (745.0 mg/l) and CB36 (1156.6 mg/l).

Table 4
Biogenic amines production by *Companilactobacillus alimentarius* strains in BC medium. The data are the mean values of three repetitions and are expressed as mg/l with the relative standard deviation.

Strains	Tyramine	Histamine	Putrescine	Cadaverine
CB1	15.1 ± 2.3	147.0 ± 21.9	ND[a]	ND
CB6	61.5 ± 4.4	ND	132.8 ± 26.1	ND
CB8	386.1 ± 16.5	ND	ND	ND
CB16	489.2 ± 28.0	647.8 ± 69.1	271.1 ± 34.3	ND
CB22	ND	ND	ND	ND
CB31	ND	ND	ND	ND
CB36	1156.6 ± 94.7	ND	ND	ND
CB41	395.9 ± 45.2	ND	ND	ND
CB43	ND	ND	ND	ND
CE49	745.0 ± 78.5	90.8 ± 22.1	ND	ND
CO12	30.1 ± 2.0	ND	ND	ND
CO24	27.0 ± 1.4	ND	ND	ND
CO50	ND	ND	ND	ND
SE14	ND	641.0 ± 71.6	ND	ND

[a] ND: not detected (under the detection limit: 3 mg/l).

Histidine decarboxylase activity was found in 4 strains. Two of them were high producers of histamine (>600 mg/l): CB16 and SE14. Minor amounts were detected for the strains CB1 and CE49, both characterized also by the presence of tyrosine decarboxylase. Putrescine was accumulated only by CB6 and CB16. Thus, this last strain was positive for the formation of three BA. Cadaverine was never detected. Finally, 4 strains did not show any decarboxylase activity (CB22, CB31, CB43 and CO50).

Species belonging to LAB are known to be the most important tyramine producer in fermented foods (Barbieri et al., 2019; Latorre-Moratalla et al., 2017). Tyrosine decarboxylase is extremely diffused and active among enterococci (Bargossi et al., 2015; Gatto et al., 2016; Ladero et al., 2012). Nevertheless, the ability to produce tyramine is a strain characteristic of many species belonging to facultatively heterofermentative lactobacilli, including *Latilactobacillus curvatus*, *Lpl. plantarum*, *Lcb. casei/paracasei*, and obligate heterofermentative lactobacilli, such as *Levilactobacillus brevis* and *Lentilactobacillus buchneri* (Barbieri et al., 2019; Marcobal et al., 2012). All these species are common constituents of the ripening microbiota of several fermented foods. Strains of the same species can also decarboxylate histidine, even if this capability is less diffused among LAB (Barbieri et al., 2019; EFSA, 2011; Landete et al., 2008; Moniente et al., 2021). Putrescine derives from the decarboxylation of ornithine, an amino acid produced by the arginine deiminase pathway (ADI). This metabolic route is particularly advantageous because it allows the production of ATP from arginine. For this reason, ADI is advantageous for microorganisms in meat environment, in which fermentable sugar are rapidly depleted (Rimaux et al., 2012). However, in some strains, ornithine can be decarboxylate producing putrescine. This ability is rare in *Lat. sakei* (Barbieri et al., 2020), but it is more diffused among other species. Few studies concerning the ability of producing BA by *C. alimentarius* are available. One strain (out of two) belonging to this species was described as a relevant histamine producer by Straub et al. (1995). In addition, a strain of *C. alimentarius* isolated from meat was able to produce cadaverine, putrescine and tyramine (Min et al., 2004) and a strain from table olives presented a tyrosine decarboxylase activity (Yalçınkaya and Başyiğit Kılıç, 2019). By contrast, no decarboxylase activity was observed in strains isolated from Himalayan fermented foods (Dewan and Tamang, 2006, 2007).

3.5. Growth kinetics in relation to temperature and NaCl concentration

Growth at different NaCl concentrations (0, 2.5 and 5 %) and temperatures (10, 20 and 30 °C) was monitored through the increase of OD_{600}. The data were fitted with the Gompertz equation as modified by Zwietering et al. (1990) to estimate the parameter *A*, μ_{max} and λ. The parameter estimated for each strain and condition and goodness-of-fit diagnostic of the regression are reported in Table S1. Fig. 1 shows the Box and Whisker plots describing the distribution of the three parameters in relation to temperature.

The median *A* value had no significant differences at 30 °C and 20 °C (2.02 and 2.05, respectively), while the μ_{max} and λ medians were strongly affected by temperature. In particular, the value of μ_{max} decreased from 0.331 to 0.186 and 0.042 OD_{600} h^{-1} passing from 30 to 10 °C and the λ median value increased from 8.50 to 14.77 and 47.63 h under the same conditions. At 10 °C one of the strains did not grow (CB31). As it is possible to observe from Fig. 1, the strains presented, at the temperatures considered, a double behaviour. At 30 °C, a first group characterized by higher growth performances, both in terms of μ_{max} and λ, included the strains CB6, CB16, CB36, CE49, CO50 and SE14. The same strains were responsible also for the best growth performances at 20 °C and 10 °C.

Concerning the effects of NaCl, the distribution of the estimated parameters is reported in the Box and Whiskers plots of Fig. 2. Increasing salt concentrations were responsible for the decrease of *A* (from a median value of 2.05 at 0 % to 1.74 at 5 %) and relevant μ_{max} decreases (0.186 OD_{600} h^{-1} at 0 % and 0.109 and 0.047 OD_{600} h^{-1} at 2.5 and 5 %, respectively). The estimation of the λ median value slightly increased from 0 to 2.5 % (from 14.77 to 17.35 h) and more drastically at 5 % (26.43 h).

As already observed for the distribution of the parameters in relation to temperature, also in the case of the effect of NaCl on *A*, the performances of the strains can be clustered into two groups, one of which was characterized by lower final *A* values. This trend was particularly evident in the samples containing the higher salt concentration (CB1, CB8, CB31, CB41, CO12 and CO24). Concerning μ_{max} the grouping effect attributable to NaCl was not evident, while it was found again in the distribution of λ. However, it is important to note that the stains showing lower *A* values did not coincide with the strains with longer λ estimates (CB6, CB16, CB36, CB43 and CE49).

Regarding pH, in Fig. 3 the Box and Whisker plots describing the parameters distribution in relation to temperature are showed, while all the data are reported in Table S2. No significant difference were observed in the pH median value decrease at 30 °C and 20 °C (approx. -2.10 units in both cases), among which CB43 and CO24 presented the more relevant pH decrease, while at 10 °C the median A_{pH} value was lower (−1.89). Significant differences were observed in μ_{pHmax} parameter, whose median value strongly decreased from 30 to 10 °C (−0.193, −0.100 and −0.030 pH h^{-1}), with high variability at 30 °C (CV 34 %). The λ_{pH} estimate parameters are almost completely superimposable with the same parameter obtained measuring OD_{600}, as demonstrated by the linear regression between the results obtained with the two methods ($\lambda_{pH} = -0.799 + 0.950 \cdot \lambda_{OD600}$, $R = 0.999$).

Concerning the effect of NaCl, *k* value and the estimated parameters were significantly influenced by its concentration (Fig. 4 and Table S2). The more relevant differences were observed in μ_{pHmax} parameter, with the strains CB1, CB22, CB31, CB43, CE49 and CO24 presenting the lower values, especially at 0 and 2.5 % NaCl. Finally, as in the case of temperature effects, also the λ_{pH} estimated for pH were related with those determined with OD_{600} ($\lambda_{pH} = -1.037 + 0.953 \cdot \lambda_{OD600}$, $R = 0.959$).

C. alimentarius is described as an environmental LAB growing between 15 and 37 °C, but information regarding its behaviour is scarce (Zheng et al., 2020). This study revealed its ability to growth at 10 °C (except for one strain) and a relatively high difference observed in the growth dynamics at the considered temperatures. Noteworthy, the growth at 10 °C can explain its presence in sausages in which low temperature can be applied during ripening. The adaptation to fermented meat environment is confirmed also by the performances showed in the presence of salt concentrations compatible with those characterizing sausages during manufacture and ripening.

4. Conclusions

C. alimentarius is a LAB species often found in fermented foods. In this

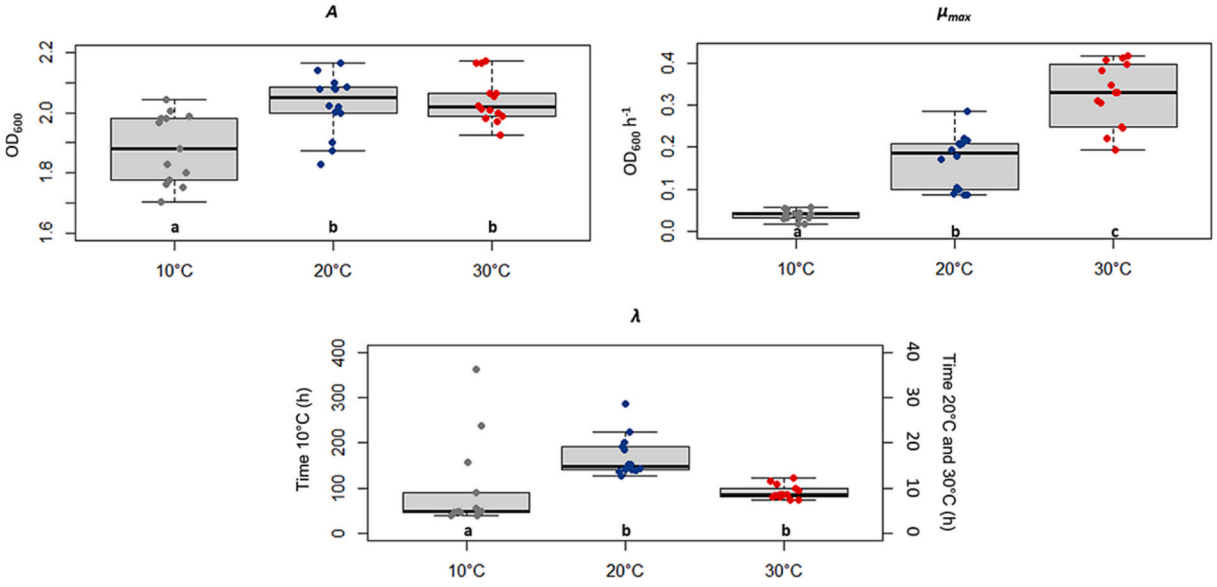

Fig. 1. Box and Whisker plots representing the distribution of parameters estimated by Gompertz eq. (*A*, μ_{max} and λ) of strain growth kinetics at different incubation temperatures (10, 20 and 30 °C). In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from median exceeds at least ±1.5 times the box height. Different letters indicate significant differences ($p \leq 0.05$) according to ANOVA.

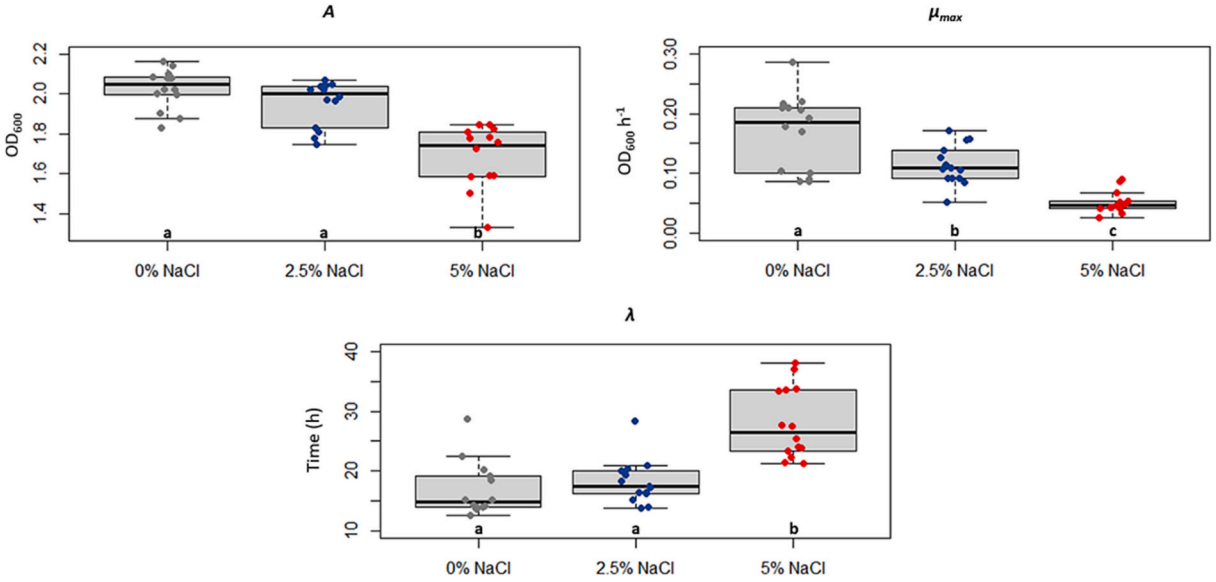

Fig. 2. Box and Whisker plots representing the distribution of parameters estimated by Gompertz eq. (A, μ_{max} and λ) of strain growth kinetics at different salt concentrations (0, 2.5 and 5 %). In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from median exceeds at least ±1.5 times the box height. Different letters indicate significant differences ($p \leq 0.05$) according to ANOVA.

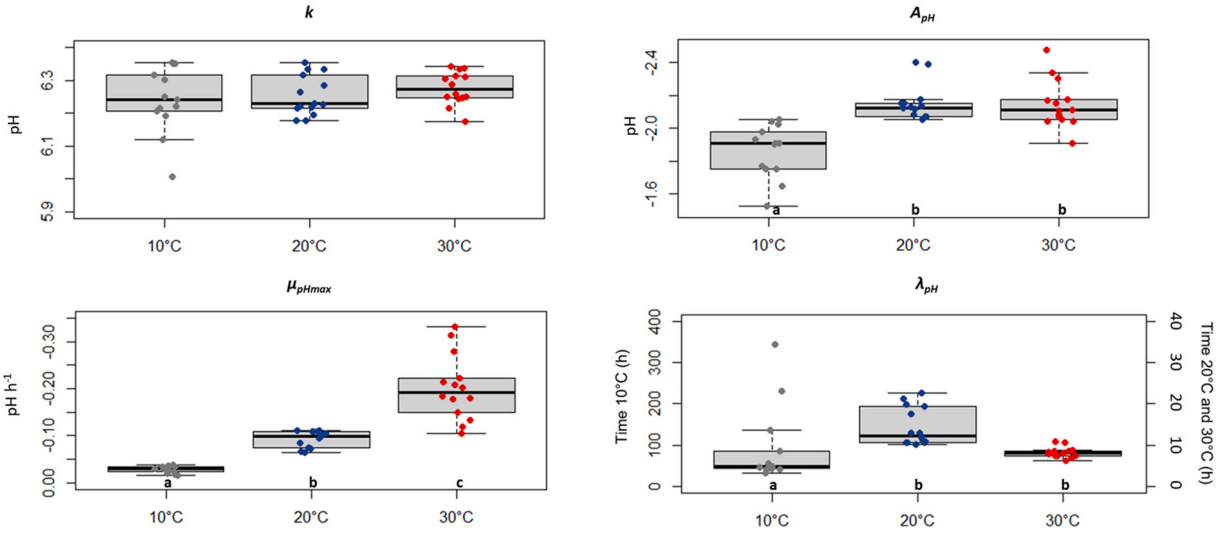

Fig. 3. Box and Whisker plots representing the distribution of parameters estimated by Gompertz equation (k, A_{pH}, μ_{pHmax} and λ_{pH}) of pH decrease at different incubation temperatures (10, 20 and 30 °C). In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from median exceeds at least ±1.5 times the box height. Different letters indicate significant differences ($p \leq 0.05$) according to ANOVA.

paper, 14 strains, previously isolated from Spanish spontaneously fermented sausages, have been characterized for their technological and safety features.

In general, a wide variability was observed in relation to the parameters considered. Among phenotypic characters, glucose was the only carbon source used by all the strains, while the utilization of other carbohydrates is a strain-dependent characteristic. Regarding safety issues, several of the tested strains were able to produce histamine, tyramine and putrescine and only one was able to express all these decarboxylating activities in relevant amounts. In addition, the antibiotic resistance greatly varied according to the strains, with the exception of vancomycin, to which all were resistant.

Concerning the technological parameters, *C. alimentarius* strains showed a relevant potential to grow in conditions of salt and temperature mimicking the level of this variables characterizing fermented sausages. Interestingly, the strains seem to show two different growth patterns, one of which characterized by lower growth potential, especially in relation to temperature.

In other words, the variability of the performances of the strains, concerning safety and technological parameters, reflects the differentiation of the phenotypic profile induced by the environmental conditions charactering the isolation source, including those derived by the raw materials.

Ultimately, among the 14 strains tested only 2 (CB22 and CB31) did not shown either decarboxylase activity or antibiotic resistance and could be candidate for a possible use as a starter culture. However, the strain CB31 was the only not able to growth at 10 °C and this could be a limitation for its use in product in which the ripening condition include

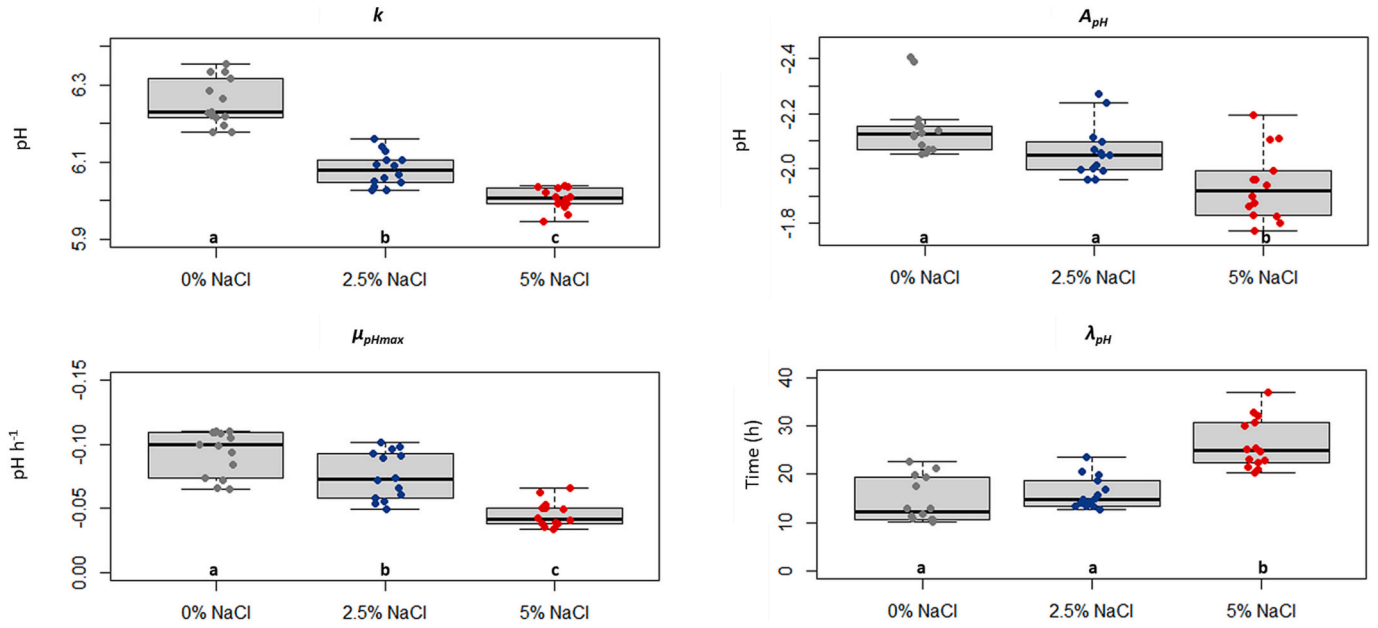

Fig. 4. Box and Whisker plots representing the distribution of parameters estimated by Gompertz equation (k, A_{pH}, μ_{pHmax} and λ_{pH}) of pH decrease at different salt concentrations (0, 2.5 and 5 %). In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from median exceeds at least ±1.5 times the box height. Different letters indicate significant differences ($p \leq 0.05$) according to ANOVA.

low temperatures. Further studies are needed to better exploit the potential of these strains including, in particular, their contribution in the accumulation of compounds affecting the aroma profile of sausages.

Given the frequent association of this species with spontaneous fermentations or ripening microbiota of various products the data presented in this study contribute to a deeper comprehension of their role, both advantageous and detrimental, in fermented foods, even in vivo trials are necessary with this purpose.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110489.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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