Properties of potentially probiotic *Lactobacillus* isolates from poultry intestines

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Abstract

The most commonly used probiotic bacteria belong to the genus *Lactobacillus*, being regarded as beneficial for poultry health and production. However, commercial probiotics do not always ensure both expected effects. In order to improve the utility properties of new preparations, the selection of new probiotic candidates should be made on the basis of the performance of the species within the poultry digestive tract. The aim of this study was to isolate and identify lactobacilli from poultry intestines, and to select probiotic candidates for subsequent *in vivo* trials. Digesta from 18 poultry specimens were collected, serially diluted, plated onto Wilkins-Chalgren anaerobe agar supplemented with 30% of rumen fluid and onto De Man, Rogosa and Sharpe agar plates, and incubated at 37 °C for 48 h under anaerobic conditions. Isolated colonies were subjected to Gram staining and catalase reaction. They were then pre-identified using matrix-assisted laser desorption ionization (MALDI) time of flight (TOF) mass spectrometry (MS). Forty-four *Lactobacillus* strains belonging to 16 species were identified and subjected to evaluations of survival under simulated gastrointestinal conditions, autoaggregation and hydrophobicity. Most of the screened *Lactobacillus reuteri* strains as well as individual strains of *L. acidophilus, L. amylovorus*, *L. gallinarum, L. ingluviei*, *L. johnsonii*, *L. oris, L. salivarius, L. saerimneri*, and *L. vaginalis* showed high survival rates under gastrointestinal tract conditions and good surface properties. The results suggest their potential for further testing as probiotic candidates in *in vivo* trials.

Lactobacillus, MALDI TOF MS, autoaggregation, hydrophobicity, probiotics

Lactobacillus populations are autochthonous residents in the gastrointestinal tracts of humans and animals, including poultry (Stephenson et al. 2010). The presence of lactobacilli in the poultry intestinal ecosystem has previously been considered as beneficial to poultry health and production (Tannock 2004). Recent studies of *Lactobacillus* species ecology and their beneficial effects have brought new data (Wang et al. 2014; Adhikari and Kwon 2017; Duar et al. 2017b), and new probiotic candidates from this group of bacteria are being sought based on current knowledge. This renewed interest is stimulated by the urgent need to reduce the consumption of antimicrobials in the poultry industry while at the same time promoting health and improving performance indicators such as mean egg weight, body weight, and the feed conversion ratio (Angelakis and Rault 2010; Olnood et al. 2015; Shokryazdan et al. 2017). Commercial probiotic preparations containing lactobacilli do not always ensure these expected effects which, among other things, may be influenced by an inappropriate process of selecting *Lactobacillus* isolates (Kizerwetter-Świda and Binek 2016). Moreover, commercial probiotic products commonly do not contain the probiotic strains stated on their labels or are even inappropriate for the stated target animal species (Lata et al. 2006; Šmídková and Čížek 2017).

Selection criteria for new probiotic candidates should be based on actual knowledge as to the importance of particular *Lactobacillus* species in poultry intestines (Adhikari and Kwon 2017; Duar et al. 2017a,b) and their probiotic functional properties. *In vitro* selection procedures usually include survival of probiotic candidates under gastrointestinal

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conditions, their surface properties, capability for competitive exclusion and antibiotic susceptibility (Bujnakova et al. 2014; Kizerwetter-Świda and Binek 2016; Rajokaa et al. 2018).

The objectives of the present study were to isolate and identify lactobacilli from poultry intestines and then to select probiotic candidates for subsequent *in vivo* trials.

Materials and Methods

Lactobacilli culture and growth conditions

Whole caeca or jejuna from 18 randomly selected healthy chickens at 4 to 40 weeks of age were processed in an anaerobic chamber (10% CO_2, 5% H_2, and 85% N_2 atmosphere; Concept 400, Baker Ruskinn, Sanford, Maine, USA) within 1 h after they were ethically sacrificed. Approximately 0.5 ml of caecal or jejunal digesta were collected and serially diluted in 4.5 ml of pre-reduced anaerobically sterilized dilution blanks and plated onto Wilkins-Chalgren anaerobe agar (Oxoid, Basingstoke, UK) supplemented with 30% of rumen fluid (Medvecky et al. 2018) and onto De Man, Rogosa and Sharpe (MRS) agar plates (Oxoid, Basingstoke, UK). All inoculated media were incubated at 37 °C for 48 h in an anaerobic chamber. Isolated colonies on the plates with the highest dilution were subjected to Gram staining and catalase reaction. They were then pre-identified using matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) mass spectrometry (MS). Colonies pre-identified as lactobacilli were subcultured on Wilkins-Chalgren anaerobe agar or MRS agar plates and the pure cultures thus obtained were used for further studies. Six strains (*L. amylovorus* CCM4380*, L. fermentum* CCM7192*, L. parabuchneri* DSM5987*, L. pentosus* CCM4619*, L. plantarum* CCM7039*,* and *L. salivarius* subsp. *salivarius* CCM7274) were included as control strains in all procedures. All cultures of lactobacilli were stored in MRS broth (Oxoid, Basingstoke, UK) with 20% glycerol at −80 °C.

Species identification using MALDI-TOF MS

Pure cultures of *Lactobacillus* spp. isolates and control strains on MRS agar plates were subjected to MALDI-TOF MS on a Microflex LT instrument (Bruker Daltonik, Germany) as described by several authors (Duskova et al. 2012; Bujnakova et al. 2014; Dec et al. 2016). Briefly, the bacterial culture on MALDI plates was overlaid with 1 µl of matrix solution containing 10 mg/ml HCCA (a-cyano-4-hydroxycinnamic acid, Sigma-Aldrich, Prague, Czech Republic) dissolved in 50% acetonitrile (Sigma-Aldrich, Prague, Czech Republic) and 2.5% trifluoroacetic acid, and then air-dried. The mass spectra were processed using the MALDI Biotyper 3.0 software package (Bruker, Leipzig, Germany) containing 6903 reference spectra, including 243 for lactobacilli. Identification was performed according to the criteria recommended by the manufacturer (ID score: 1.700–1.999 probable genus identification; 2.000–2.299 secure genus identification, probable species identification; 2.300–3.000 highly probable species identification).

Tolerance to gastrointestinal conditions

Tolerance of isolates to simulated gastric juice and bile salts was assayed as described by Jena et al. (2013). Briefly, for the bile tolerance test, MRS broth (Oxoid, Basingstoke, UK) was supplemented with 0.3% (w/v) bile salts (Sigma-Aldrich, Prague, Czech Republic). Simulated gastric juice contained 6.4 g $NaHCO_3$, 0.239 g KCl, 1.28 g NaCl, and 0.1% (w/v) pepsin (Sigma-Aldrich, Prague, Czech Republic) per litre of MRS broth (pH 2.5). For both tests, 5 ml of lactobacilli culture grown overnight in MRS broth at 37 °C were pelleted and washed twice with 4 ml of phosphate buffered saline (PBS; pH 7.2). Subsequently, culture density was adjusted to McFarland turbidity standard 1.0 (Densi-La-Meter®, Erba Lachema, Brno, Czech Republic) (ca 3×10^8 cfu/ml) and inoculated into 10 ml of modified MRS broth containing bile salts or simulated gastric juice. Samples were then incubated at 37 °C for 30, 60, 90, 120, or 180 min. The number of viable cells was assessed by a serial dilution and plate count method. The results were expressed as growth rate (%) of *Lactobacillus* strains.

Autoaggregation assay

Autoaggregation capabilities were assessed according to the procedure described by Collado et al. (2007) with minor modifications. The lactobacilli culture grown overnight in MRS broth at 37 °C was pelleted, washed twice with PBS, and then resuspended in PBS. The optical density (OD) of the bacterial suspension was adjusted to McFarland turbidity standard 2.0 (Densi-La-Meter®, Erba Lachema, Brno, Czech Republic). Bacterial cell suspensions were incubated without agitation in closed measuring cuvettes (2 ml) at room temperature (22 ± 2 °C) for different time periods (0, 2, 4, 6, 24, and 48 h). At the determined time the absorbance was measured at 600 nm using a Biowave Cell Density Meter (WPA, Cambridge, UK). Autoaggregation assay was accomplished in one experiment with each isolate and was expressed as autoaggregation percentage ($A\% = 1 - (A_t/A_0)/100$), where A_t represents absorbance at the determined time and A_0 the absorbance at t_0.

Cell surface hydrophobicity assay

The degree of hydrophobicity of the cultures was specified according to procedures described previously (Rosenberg 1984; Jena et al. 2013) and which are based on affinity of bacterial cells for toluene

Table 1. *Lactobacillus* control strains and isolates of poultry origin, abundance in section of the gut (J – jejunum, C – cecum) in $\log_{10}$ counts, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS) ID scores.

Lactobacillus spp.	Strain No.	Section of gut	$\log_{10}$ counts	ID score	*Lactobacillus* spp.	Strain No.	Section of gut	$\log_{10}$ counts	ID score
L. acidophilus	275	C	8	1.891	*L. oris*	316	C	ND	1.682
L. acidophilus	343	J	7	2.322	*L. reuteri*	21	C	9	2.212
L. agilis	358	J	8	2.368	*L. reuteri*	28	C	9	2.216
L. agilis	650	C	ND	2.331	*L. reuteri*	71	C	10	2.392
L. amylovorus	296	C	2	1.761	*L. reuteri*	166	C	9	2.363
L. aviarius	347	J	7	2.439	*L. reuteri*	554	J	8	2.286
L. coleohominis	574	C	8	1.937	*L. reuteri*	694	C	ND	2.191
L. crispatus	336	J	7	2.325	*L. salivarius*	34	C	9	2.421
L. crispatus	724	C	6	2.128	*L. salivarius*	48	C	10	2.489
L. gallinarum	101	C	8	2.385	*L. salivarius*	60	C	10	2.353
L. gallinarum	117	C	9	2.240	*L. salivarius*	84	C	10	2.301
L. gallinarum	153	C	10	2.316	*L. salivarius*	128	C	9	2.315
L. gallinarum	304	C	ND	2.101	*L. salivarius*	321	C	3	2.101
L. gallinarum	315	C	4	2.342	*L. salivarius*	453	C	7	2.284
L. gallinarum	447	C	7	2.198	*L. salivarius*	647	C	ND	2.250
L. gasseri	197	C	9	2.585	*L. saerimneri*	299	C	ND	2.115
L. ingluviei	443	C	8	1.984	*L. saerimneri*	374	C	8	2.073
L. ingluviei	593	C	ND	2.086	*L. vaginalis*	337	J	7	2.055
L. ingluviei	681	C	ND	2.127	*L. vaginalis*	683	C	ND	1.967
L. johnsonii	171	C	9	2.317	*L. amylovorus*	CCM4380			2.392
L. johnsonii	297	C	ND	2.092	*L. fermentum*	CCM7192			1.915
L. johnsonii	340	J	8	2.396	*L. parabuchneri*	DSM5987			2.148
L. kitasatonis	294	C	7	1.771	*L. pentosus*	CCM4619			2.387
L. kitasatonis	458	C	7	2.232	*L. plantarum*	CCM7039			2.440
L. oris	301	C	ND	1.589	*L. salivarius* subsp. *salivarius*	CCM7274			2.273

ND – not done

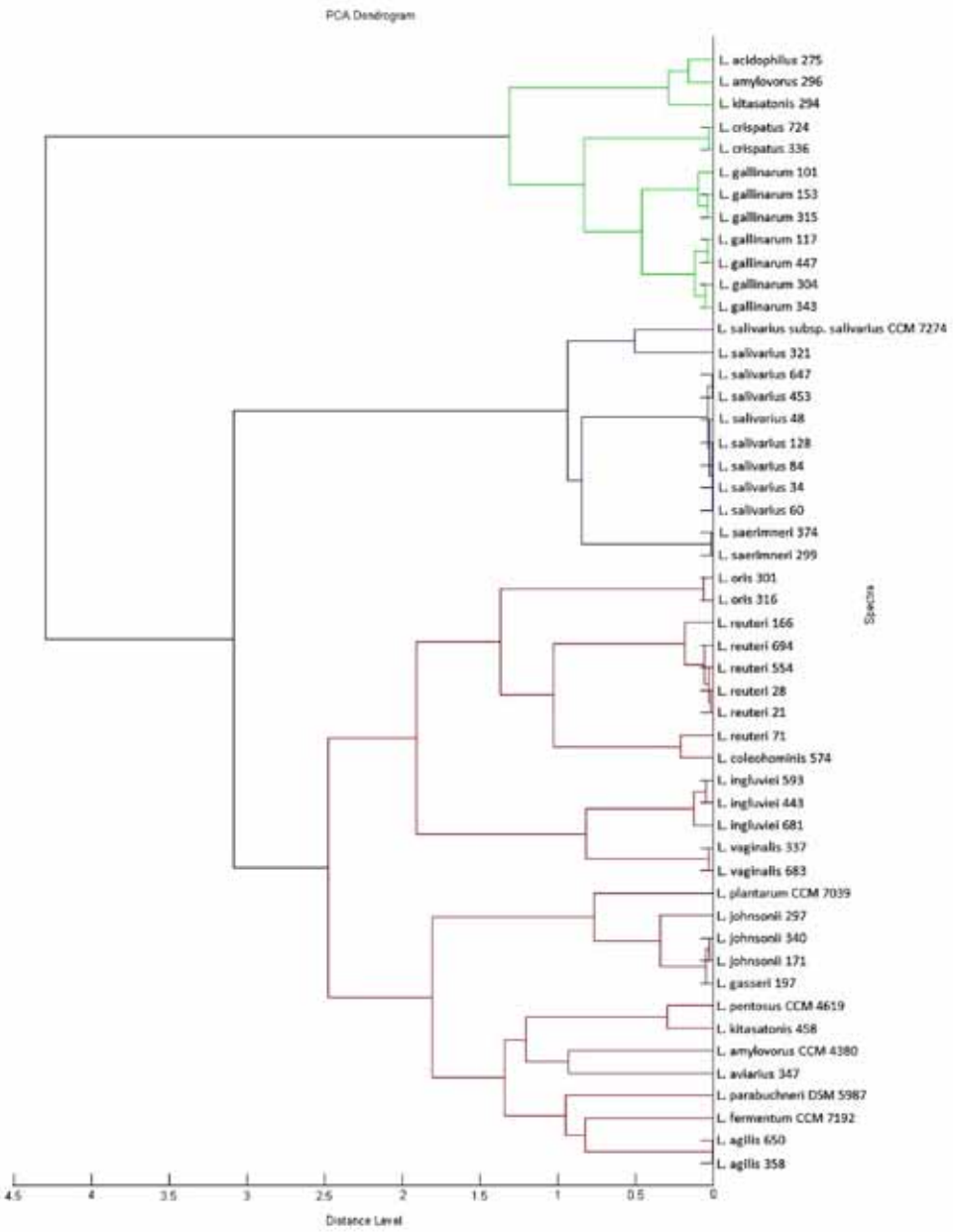

Fig. 1. Main-spectra dendrogram of matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) profiles generated by the MALDI Biotyper.

in a two-phase system. Hydrophobicity was calculated from three replicates as the percent decrease in OD of the original bacterial suspension due to cells partitioning into the hydrocarbon layer. The cell surface hydrophobicity (%) of isolate adhering to hydrocarbon solvent was calculated according to the following equation: Hydrophobicity % = OD660 before mixing − OD660 after mixing/OD660 before mixing × 100.

Data processing

Bacterial counts were represented as the average logarithm of colony forming units (CFU) or the average values of absorbance (OD). All data were entered into spreadsheets (Excel, Microsoft) that were used to calculate the percentage of autoaggregation, hydrophobicity, and viability of *Lactobacillus* strains in simulated gastrointestinal conditions.

Results

Identification of lactobacilli

The isolates were pre-identified by Gram staining, catalase reaction, and application of MALDI TOF MS. Forty-four *Lactobacillus* isolates belonging to 16 species were obtained from intestinal contents of 18 poultry intestinal specimens. Table 1 summarizes the *Lactobacillus* species pre-identified by MALDI TOF MS and their abundance in different intestinal compartments. A dendrogram designed on cluster analysis of MALDI-Biotyper protein mass spectra of these isolates showing distinctive clusters consisting of the same *Lactobacillus* species along with control strains is presented as Fig. 1. Corresponding ID score values for the same set of lactobacilli are included in Table 1. The ID scores for 8 strains were between 1.589 and 1.999, and for 36 strains they ranged from 2.000 to 2.489. Despite low ID values for 2 isolates of *L. oris* (1.589 and 1.682), the application of MALDI TOF MS allowed identification at species level for 82% of the isolates and for 14% of the isolates provided ID values at genus level.

Tolerance to simulated gastric juice

The effect of simulated gastric juice on the growth rate of lactobacilli was evaluated for 44 isolates of poultry origin and 6 control strains. Differences were found among *Lactobacillus* strains in their tolerance to simulated gastric conditions (Fig. 2). After 30 min of the treatment, the following isolates lost their viability: *L. agilis* 358, 650, *L. kitasatonis* 458, *L. parabuchneri* DSM5987, *L. pentosus* CCM4619, and *L. plantarum* CCM7039. Generally, a low level of viability was recorded for control strains. By contrast, all strains of *L. reuteri* and one strain of each *L. acidophilus* (275), *L. gasseri* (197), *L. oris* (316), and *L. vaginalis* (683) showed high levels of viability through time.

Effect of bile salts

Small intestine conditions were simulated in MRS broth with 0.3% bile salts. The 44 *Lactobacillus* strains and 6 control strains were tested for ability to grow in these conditions. The most rapidly decreasing growth rates were recorded for the following individual strains: *L. agilis* 650; *L. gallinarum* 101, 117, and 153; *L. oris* 301, and *L. amylovorus* CCM4380. The remaining strains, e.g. *L. amylovorus* 296, *L. aviarius* 347, *L. coleohominis* 574, *L. crispatus* 336, *L. ingluviei* 681, or *L. saerimneri* 374, exhibited slight diminished or even increased growth rate (Fig. 3).

Autoaggregation and hydrophobicity assay

The percentage of autoaggregation increased with time for each *Lactobacillus* strain. With the exceptions of strains *L. gasseri* 197, *L. johnsonii* 340, *L. coleohominis* 574, *L. vaginalis* 337, and *L. fermentum* CCM 7192, most of the strains (61%) were highly positive in both tests. Positive relationship between autoaggregation percentage and hydrophobicity percentage was evident in most *Lactobacillus* strains (91%, n = 44). Conversely, negative link was proven in the strains *L. reuteri* 21, *L. gasseri* 197, *L. gallinarum* 117, and *L. fermentum* CCM 7192. The results of autoaggregation and hydrophobicity assays are summarized in Fig. 4.

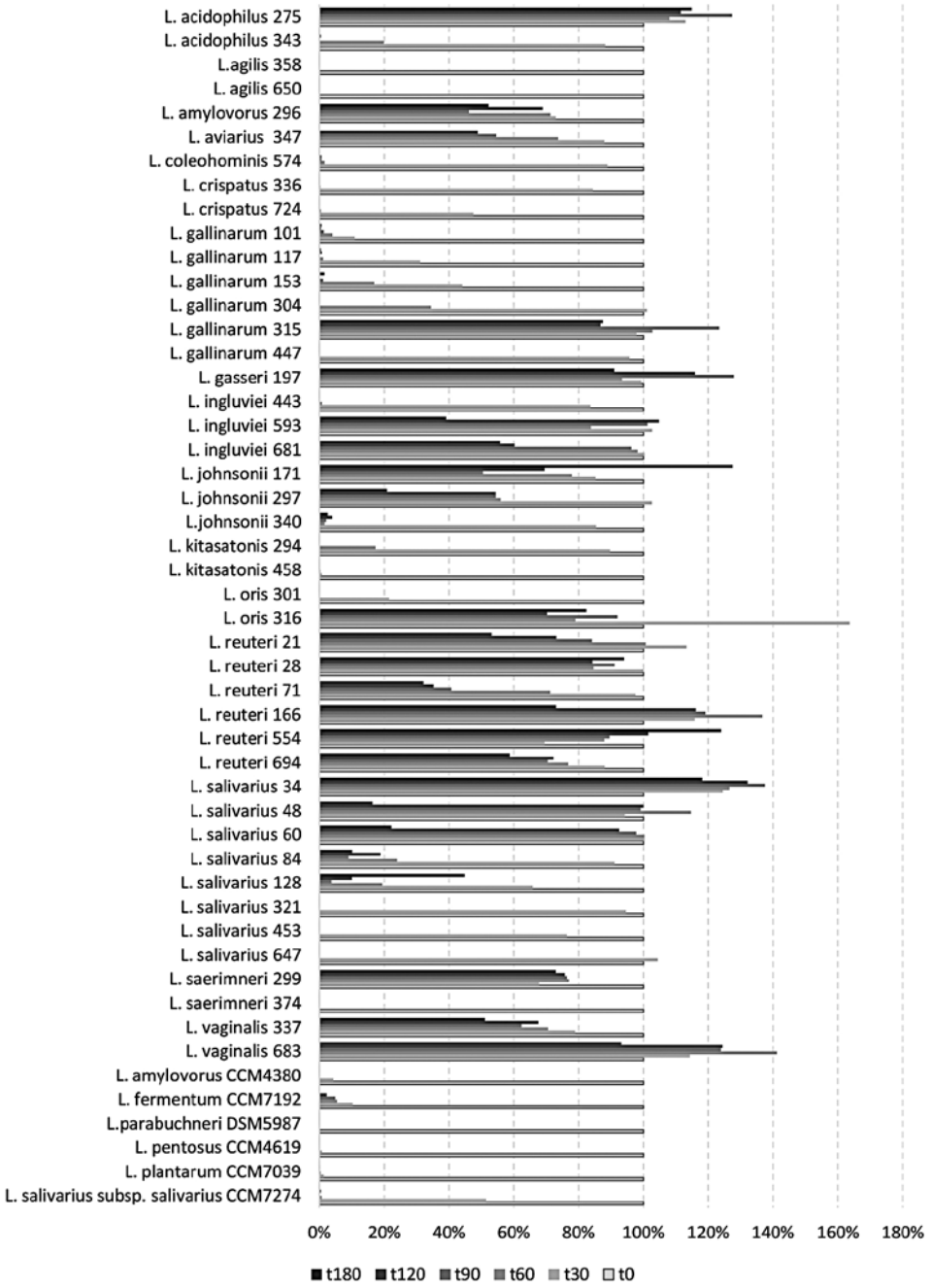

Fig. 2. *Lactobacillus* strains growth rate (%) after incubation in the Man, Rogosa and Sharpe (MRS) broth with 0.1% pepsin (pH 2.5) at 37 °C for 30, 60, 90, 120, and 180 min.

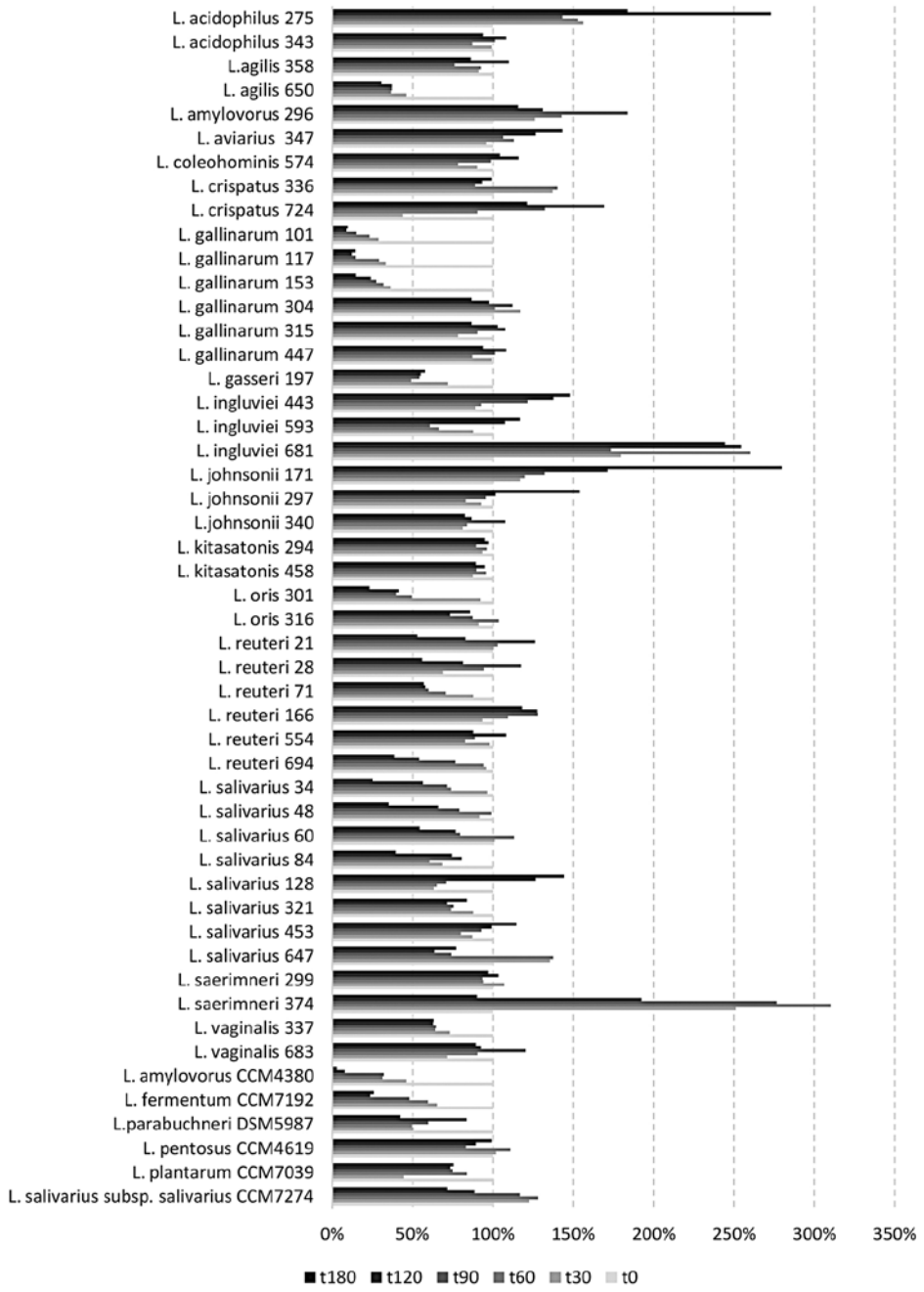

Fig. 3. *Lactobacillus* strains growth rate (%) after incubation in the Man, Rogosa and Sharpe (MRS) broth with 0.3% bile salts at 37 °C for 30, 60, 90, 120, and 180 min.

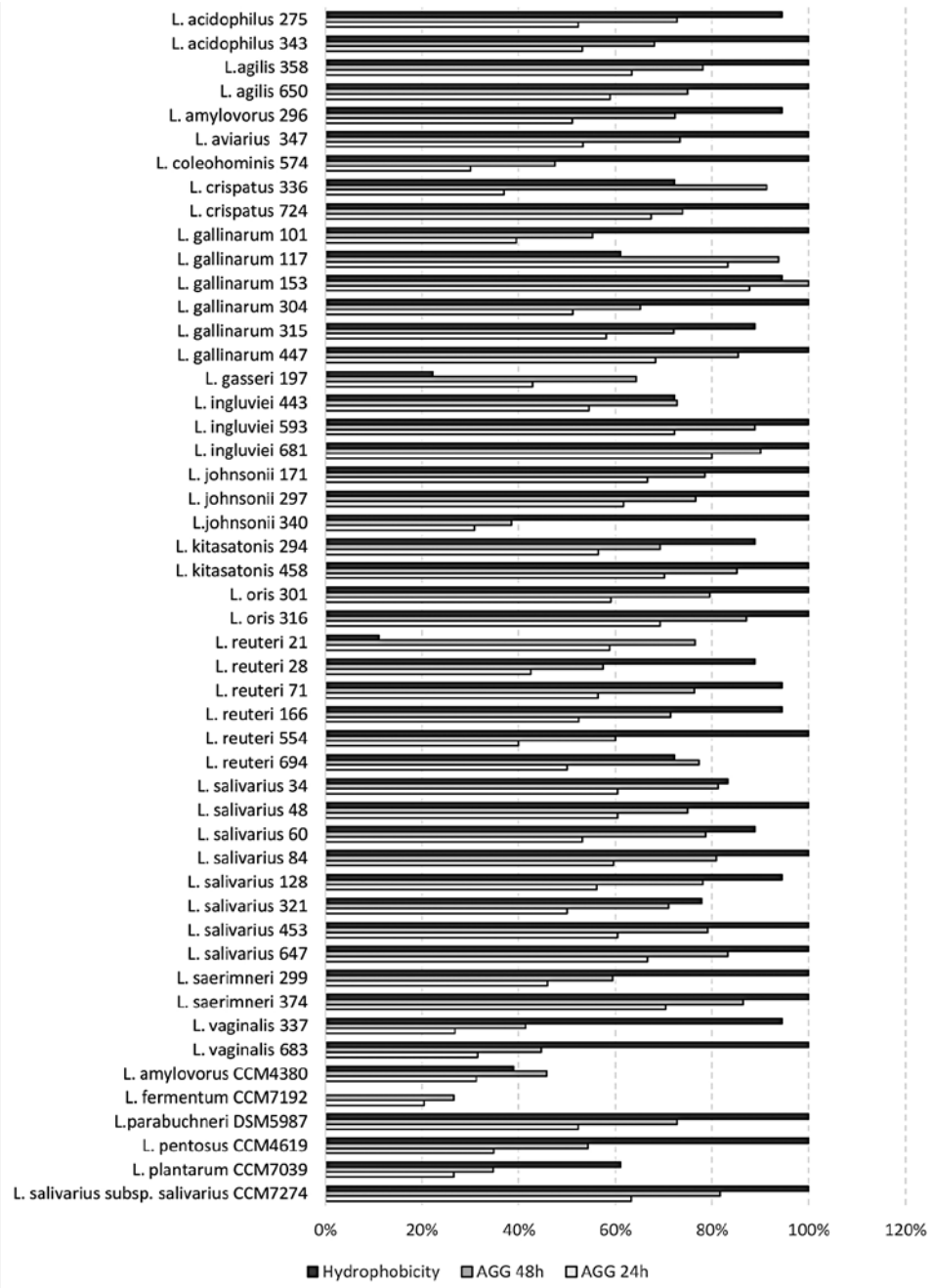

Fig. 4. Autoaggregation (AAG) and hydrophobicity percentages for *Lactobacillus* strains of poultry origin after incubation at 22 ± 2 °C.

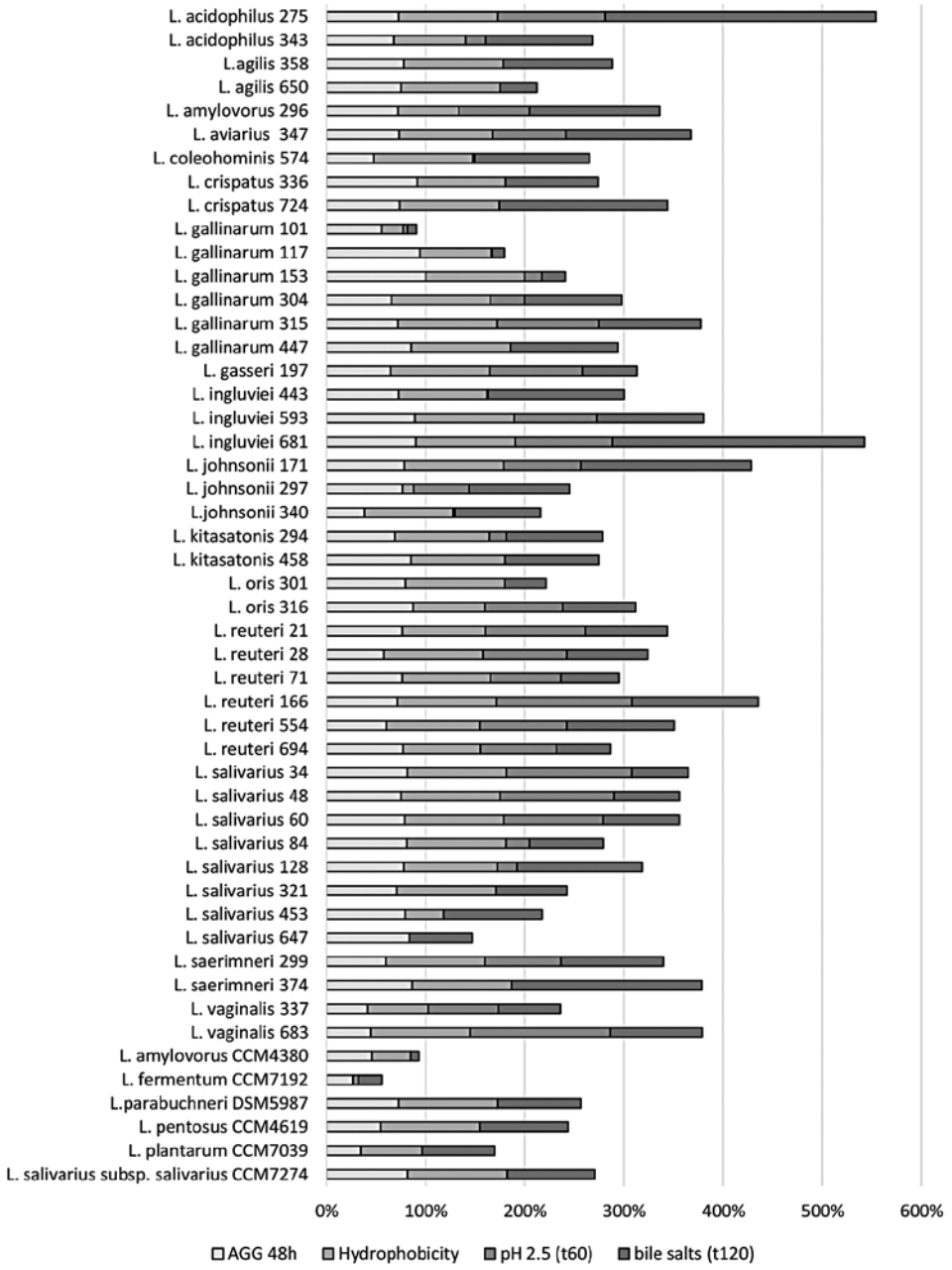

Fig. 5. Cumulative percentages of autoaggregation (AAG) after 48 h, hydrophobicity after 120 min, growth rate after incubation in MRS with 0.1% pepsin (pH 2.5) for 60 min and MRS with 0.3% bile salts at 37 °C for 120 min for *Lactobacillus* strains.

Discussion

Concentrated effort to limit the consumption of antibiotics in the poultry industry is leading to the introduction of alternative approaches to protecting animal health while improving production. One such approach is to take advantage of the new-generation probiotics that will be selected by appropriate *in vitro* and *in vivo* procedures (Ahasan et al. 2015). The most commonly used probiotic bacteria belong to the genus *Lactobacillus.* New ecological, genomic, and phylogenomic data about *Lactobacillus* species of poultry origin have recently become available (Stephenson et al. 2010; Wang et al. 2014; Adhikari and Kwon 2017; Duar et al. 2017a). In addition, new evidence indicates high levels of niche conservatism among such bacteria, with lifestyles ranging from free-living to strictly symbiotic species (Duar et al. 2017b). These aspects should be taken into account when selecting probiotic candidates.

Application of MALDI TOF MS for lactobacilli identification has proven itself in several previous studies (Duskova et al. 2012; Bujnakova et al. 2014; Dec et al. 2016), mostly in combination with genotypic methods. Although genotypic and phenotypic characterization of lactobacilli still poses several difficulties (Foschi et al. 2017), in our study high discriminatory power (82%) of MALDI-TOF MS for identifying *Lactobacillus* species of poultry origin was proven. This procedure was sufficient for the selection of candidate probiotic strains.

Among our *Lactobacillus* isolates, three species (*L. gallinarum*, *L. reuteri*, and *L. salivarius*) were most widely represented. The spectrum of *Lactobacillus* species detected was comparable to those from previous studies (Stephenson et al. 2010; Wang et al. 2014; Adhikari and Kwon 2017). The presence of lactobacilli is commonly most dominant in the digesta of the chicken's small intestine, and therefore their high abundance in our caecal samples was somewhat surprising.

Since probiotics are commonly administered *per os*, they must be able to tolerate the passage through the stomach and to survive in the small intestine. Therefore, resistance to the gastric juice in the stomach and resistance to the bile salt in the small intestine are important selection criteria for probiotic candidates (Giraffa 2012). In our study, all strains of *L. reuteri* and *L. ingluviei*, as well as individual strains of other species showed a high degree of tolerance to the simulated gastric conditions. When evaluating this attribute, it is possible to determine simultaneously whether bile salt resistance is exhibited by the same strains. This necessary precondition was met by all strains of *L. reuteri,* as well as by *L. acidophilus 275, L. amylovorus* 296, *L. gallinarum* 315*, L. gasseri* 197, *L. ingluviei* 681, *L. johnsonii* 171, *L. oris* 316*, L. salivarius* 34*, L. saerimneri* 299, and *L. vaginalis* 683.

Aggregation among bacterial cells of the same strain (autoaggregation) is an important and advantageous property helping bacteria to thrive within several ecological niches. Aggregating bacteria may achieve adequate mass to form biofilms or adhere to mucosal surfaces of the intestine and survive there (Grześkowiak et al. 2012). Although the interactions between microbial and host cells are non-specific, there is a good correlation in probiotic strains between their surface hydrophobicity and the ability to adhere to the intestinal mucosa (Wadström et al. 1987). A hydrophobicity test was utilized as an indirect method for additionally assessing the probability that probiotic candidates will be able to adhere to the intestinal mucosa cells and estimating their prospective capability for intestinal colonization. With the exceptions of the strains *L. reuteri* 21, *L. gasseri* 197, and *L. gallinarum* 117, our results showed positive correlation between the capabilities for autoaggregation and hydrophobicity.

Fourteen strains selected from the 44 investigated *Lactobacillus* strains (32%) complied with the evaluation criteria for potential probiotic candidates (Fig. 5). Not all strains of given *Lactobacillus* species exhibit the same profile as indicated by these evaluation

criteria, thereby confirming that these properties are strain-specific and not shared by all strains of the same species.

In conclusion, our results indicate that some *Lactobacillus* strains can be classified as probiotic candidates due to their tolerance for simulated gastrointestinal conditions and surface properties associated with intestinal colonization. They can therefore be included into *in vivo* trials for testing health- and performance-related functional properties on a poultry model. Additional *in vitro* studies would be then required to confirm the strains' capability for competitive exclusion and antibiotic susceptibility, as well as to assess their stability within manufacturing processes and therapeutic application forms.

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