ANTAGONISTIC ACTIVITY OF NEWLY ISOLATED *LACTOBACILLUS* STRAINS AGAINST *MORGANELLA MORGANII*

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Abstract. *Morganella morganii* is an important clinical pathogen with fast-paced multidrug resistance and virulence. Probiotics with potent antimicrobial activity are considered as a promising alternative to antibiotics in infection treatment. We isolated 12 lactobacilli strains of human and plant origin and characterized their beneficial properties focusing on their antagonistic activity against *M. morganii*. Tolerance to the hostile gastrointestinal environment, surface properties (hydrophobicity and autoaggregation), and acidification rate values varied considerably between strains and were strain-specific. Most *Lactobacillus* strains showed antibiotic resistance profiles typical for lactobacilli. Lactobacilli demonstrated inhibitory activity towards the growth of *M. morganii* in the agar-overlay assay, produced bacteriocins and coaggregated with *M. morganii* cells, but did not affect the growth of the pathogen during co-culturing in the mixed-species biofilms. *Lactiplantibacillus plantarum* strain FCa3L was selected as the candidate strain with potential probiotic properties for further investigation.

Keywords: Lactobacilli, *Morganella morganii*, Probiotic, Antagonism.

List of Abbreviations

BHI – Brain heart infusion
CFU – colony forming unit
CBP – crude bacteriocin preparation
MRS – de Man-Rogosa-Sharpe
GIT – gastrointestinal tract
LPS – lipopolysaccharide
LB – lysogeny broth
MALDI-TOF – matrix-assisted laser desorption/ionization – time of flight
MATH – microbial adhesion to hydrocarbons
PBS – phosphate-buffered saline
TTA – total titratable acidity
R – resistant
MS – moderately susceptible
S – susceptible

Introduction

Morganella morganii is an important opportunistic pathogen, which can cause a wide variety of community-acquired and nosocomial infections. This enterobacterium has been reported as a cause of catheter-associated bacteriuria, complex infections of the urinary and/or hepatobiliary tracts, wound infection, peritonitis, central nervous system infection, and septicaemia. *M. morganii* infections are associated with a high mortality rate, although in most cases they are suitable for treatment by appropriate antibiotic therapy (Liu *et al*., 2016; Zaric *et al*., 2021). *M. morganii* is commonly found in the environment and in the normal human microbiota. Patients who suffer from *M. morganii* bacteremia are usually immunocompromised, diabetic, or elderly, and/or have at least one serious underlying disease. Virulence factors of *M. morganii* include urease, hemolysins, and lipopolysaccharide (LPS) (Liu *et al*., 2016). Besides, in recent years the drug resistance and virulence of *M. morganii* has seriously increased (Bandy, 2020). *M. morganii* is not a common pathogen to produce biofilm. Ganderton *et al*. (1992) examined bacterial biofilm in 50 Foley bladder catheters collected from patients undergoing long-term catheterization and found that one of the thinnest biofilms observed was from the catheter colonized by M. morganii. Yet, annotated *M. morganii* genomes have revealed genes corresponding to biofilm formation (Chen *et al*., 2012; Minnullina *et al*., 2019). There is an individual case of chronic osteomyelitis caused by biofilm producing strain of *M. morganii* (De *et al*., 2016).

Lactobacilli (former taxonomic group *Lactobacillus*) is one of the most important micro-

bial genera that are generally used as probiotics (Ilinskaya *et al*., 2017). The latter are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (WHO-2001). Several probiotic lactobacilli strains have been found to be effective in prevention and treatment of infections caused by *Clostridioides difficile*, *Helicobacter pylori*, EHEC, *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Staphylococcus aureus*, *Candida albicans*, and *Candida glabrata* (Silva *et al*., 2020). Antimicrobial action of lactobacilli is based on several mechanisms, such as modulation of the immune response; competition with pathogens for binding sites and nutrients; declination of intestinal luminal pH via production of lactic acid, acetic acid, formic acid, and other organic acids; secretion of antimicrobial substances such as bacteriocins, hydrogen peroxide, ethanol, and fatty acid (Servin, 2004; Zhang *et al*., 2018).

Although lactobacilli and *M. morganii* inhabit the same niches including human gastrointestinal and urogenital tract, data about their interactions are very limited. The inhibitory effect of neutralized cell free culture supernatants of some *Lactobacillus* strains was demonstrated in several studies, thus proposing their ability to produce bacteriocins effective against *M. morganii* (Djaafar *et al*., 1996; Abdulla *et al*., 2014). Conversely, the cell free culture supernatants of three *L. acidophilus* strains were not able to inhibit *M. morganii*, though were effective against several enteropathogens (Singh *et al*., 2004). The aim of the current study was to characterize the probiotic potential of twelve newly isolated *Lactobacillus* strains and to assess their antagonistic activity against *M. morganii*.

Materials and Methods

Isolation of Lactobacilli and Growth Conditions

The *Lactobacillus* strains used in this study are listed in Table 1. All lactobacilli were grown in de Man-Rogosa-Sharpe (MRS) broth or MRS agar (HiMedia, India) at 37 °C unless otherwise indicated. For anaerobic cultivation Anaerogas gaspack (NIKI MLT, Russia) was used.

Colon samples were obtained from five patients undergoing colorectal surgery in the Republican Clinical Hospital of the Ministry of Health of the Republic of Tatarstan between September and December 2015. The study was approved by the Local Ethics Committees (Protocol No. 8, Kazan Federal University, 05.05.2015; Protocol No. 9, Kazan State Medical University, 24.11.2015). Informed consent was given by all the patients enrolled in this study. For isolation of lactobacilli from colon tissues, about 0.5 g of each sample was thoroughly washed with 10 mL of sterile physiological solution (0.85 g NaCl in 100 mL of milliQ water) for 30 min at 37 °C and 180–200 rpm. Then serial 10-fold dilutions in sterile phosphate-buffered saline (PBS: 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium phosphate, 1.8 mM monopotassium phosphate, pH 7.4) were prepared and subsequently plated onto MRS agar followed with incubation under anaerobic conditions for 48 h.

Isolation of lactobacilli from fecal samples collected from healthy volunteers (1 sample per person) was performed as described in our previous study. Briefly, about 5 g of each sample was cultured in 45 mL of MRS broth and incubated under anaerobic conditions for 24 h. Further dilution and plating of the resulting enrichment culture was performed as described before for colon samples.

For the isolation of lactobacilli, sauerkraut was collected from a local market (Kazan, Tatarstan, Russia). About 5 g of each sample was vigorously blended with 50 mL of PBS containing 5 g of sterile sand. The resulting suspension was used to inoculate 50 mL of MRS broth (1:9), followed by incubation for 24 h. The enrichment culture was 10-fold diluted with PBS and spread-plated onto cabbage agar (CA) (cabbage 200 g, glucose 20 g, peptone 10 g, agar 20 g, water 1000 mL) with chalk ($CaCO_3$, 3%). The plates were incubated under anaerobic conditions for 48 h. The bacterial colonies that developed clear zones were considered as putative lactic acid bacteria and were individually

picked and streaked on MRS agar plates (Anisimova *et al*., 2017).

The suspected colonies were tested by Gram staining, catalase, and endospore forming tests, and only those that were Gram-positive, cocci or rod-shaped, non-spore forming, and catalase-negative were selected for further studies. The strains were maintained as stab cultures in MRS agar for immediate use and in 20% glycerol for storage at – 80 °C. Prior to all experiments bacterial cultures were propagated twice in MRS broth at 37 °C under microaerophilic conditions.

Identification of Lactobacilli

For species identification, MALDI-TOF mass spectrometry (Bruker Biotyper system, Bruker Daltonics, Germany) was applied, as previously described (Foschi *et al*., 2017). Briefly, single fresh colonies from MRS agar were smeared in two replicates onto a ground steel target (Bruker Daltonik), overlaid with 1 μL of a saturated solution of alpha-cyano-4-hydroxycinnamic acid matrix solution in 50% acetonitrile and 2.5% trifluoroacetic acid, and air dried at room temperature. Mass spectra recorded according to the manufacturer's instructions were compared with the ones in the integrated reference database (version 3.2.1.1). Log scores ≥ 2.0 were accepted for reliable species assignment; scores comprised in the range 1.7–2.0 indicated identification at the genus level; and scores under 1.7 were considered unreliable identification.

M. morganii strains and growth conditions

The *M. morganii* strains (MM1, MM4, MM190) were isolated from the urine of a 59-year-old man, 35-year-old woman, and a 3-year-old boy, respectively, who had community-acquired urinary tract infections. Samples for MM1 and MM4 were collected in October 2014, whereas that of MM190 was taken in June 2015 by the Clinical Diagnostic Laboratory Biomed (Kazan, Republic of Tatarstan, Russia) (Minnullina *et al*., 2019). Bacteria were incubated aerobically at 37 °C in Lysogeny broth and Lysogeny agar (LB medium) (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 8.5).

Tolerance to simulated human GI tract

Acid and bile tolerances of lactobacilli were carried out as described in (Gavrilova *et al*., 2019). Synthetic gastric fluid (0.83% proteose peptone, 0.35% D-glucose, 0.205% NaCl, 0.06% KH2PO4, 0.011% CaCl2, 0.037% KCl, 0.005% porcine bile, 0.01% lysozyme, 0.001% pepsin, pH 2.5) was used to simulate hostile conditions of the gastrointestinal tract (GIT) (Beumer *et al*., 1992).

Cell surface hydrophobicity

The degree of hydrophobicity was evaluated using the Microbial Adhesion To Hydrocarbons (MATH) method with n-hexadecane, as previously described in (Kirillova *et al*. 2017). Strains were classified according to their hydrophobicity capacities according to (Li & McLandsborough, 1999)

Antibiotic resistance

Antibiotic resistance was assessed by the disk diffusion method, as described earlier (Anisimova & Yarullina, 2019). Antibiotic discs were from Scientific Research Centre of Pharmacotherapy (St. Petersburg, Russia). Strains were classified either as resistant (R), moderately susceptible (MS), or susceptible (S) based on zones of growth inhibition according to (Melo *et al*., 2017).

Antagonistic activity

Antagonistic activity was examined by agar spot test described in (Schillinger & Lücke, 1989), with modifications. Briefly, overnight cultures of individual *Lactobacillus* strains were spotted (3 μL) on the surface of MRS agar and incubated anaerobically (Anaerogas gaspack, NIKI MLT, Russia) for 24 h at 37 °C to develop the spots. Then, the plates were overlaid with semi-hard LB medium (0.7% agar) inoculated with 100-μL volume of an overnight cultures of individual *M. morganii* strains. After 24 h of aerobic incubation, zones of bacterial growth inhibition were measured from the edge of the colony to the edge of the inhibition zone. The inhibitory effect of MRS was used as negative control.

Bacteriocin production

Production of bacteriocins was detected in agar spot test as described in (Schillinger & Lücke, 1989). In contrast to the above described method, MRS agar contained only 0.2% glucose. Low glucose concentration was used in order to inhibit lactic acid fermentation and to exclude the inhibitory effect of organic acids. Anaerobic conditions of incubation were used to minimize the formation of hydrogen peroxide and acetic acid. Inhibition was scored positive if the width of the clear zone around the *Lactobacillus* colonies was 0.5 mm or larger.

For purification of bacteriocins, the *Lactobacillus* strains were grown in MRS broth at 37 °C for 24 h until the early stationary phase. The cultures were centrifuged at 5,000 rpm for 10 min and the supernatants were saturated with 70% ammonium sulphate and stored at 4 oC for 18-20 h to precipitate out the proteins. After centrifugation at 13,000 rpm at 4 oC for 10 min, the pellet was collected and dissolved in 100 µL deionized water. This solution was designated as the crude bacteriocin preparation (CBP). 5 µL of CBPs were spotted onto lawns of *M. morganii* MM190 made on LB plates. After incubation at 37 °C for 18-24 h, LB plates were observed for appearance of inhibitory zones (Zheng & Slavik, 1999).

Acidification rate

The acidification rate of the *Lactobacillus* strains was evaluated by measuring pH and Total Titratable Acidity (TTA) in the cell-free supernatants. For that, 20 mL of MRS broth were inoculated with the overnight cultures (1% v/v) of individual *Lactobacillus* strains and incubated for 48 h at 37 °C. Then cultures were centrifuged at 5,000 rpm for 10 min and pH (pH-150MI, Russia) and TTA were measured in the resulting supernatant.

Intensity of acidogenesis (Ia) was calculated by the formula: Ia = (pH – pH0)/A600, where pH and A600 are pH and absorbance at 600 nm of the 48 h culture in MRS broth, respectively, and pH0 is pH of the MRS broth before inoculation (Anisimova *et al*., 2017).

To determine TTA, 10 mL of cell-free supernatant was diluted with 20 mL of distilled water and titrated with 0.1 N NaOH using phenolphthalein as an indicator. TTA (mmol acid/g or mL) was calculated by multiplication of NaOH needed for titration by 0.09 (Yang *et al*., 2012).

Autoaggregation and coaggregation assays

Autoaggregation abilities were measured as described in (Kos *et al*., 2003). Briefly, the overnight cultures were centrifuged at 5,000 rpm for 10 min, the pellet was washed twice with PBS and then resuspended in PBS to an optical density of 0.5 at 600 nm (A_0) (approximately 10^7–10^8 CFU/mL). Bacterial cell suspensions (4 mL) were incubated at room temperature in tubes for 4 or 24 h without shaking. The aqueous phase was gently taken out to measure its absorbance at 600 nm (A_1). Autoaggregation percentage was calculated as $(1 - A_1/A_0) \times 100$.

In the coaggregation test, equal volumes (2 mL) of *Lactobacillus* and *M. morganii* MM190 cells were mixed and incubated for 4 h at room temperature without agitation. The absorbances (A600 nm) were measured for the mixture and for the bacterial suspensions alone. Coaggregation was calculated as: $[(A_x + A_y) - 2\times A_{mix}]/(A_x + A_y) \times 100$, where A_x and A_y represent absorbances at 600 nm of the separate bacterial suspensions, A_{mix} represents the absorbance of the mixed bacterial suspension.

Biofilms assay

To assess biofilm formation, *M. morganii* MM190 and individual *Lactobacillus* strains were cultured together in 24-well polystyrene cell culture microplates (flat bottom, Eppendorf, no. 730.011) in brain heart infusion medium (BHI, BD Bacto) supplemented with 0.005% manganese sulfate and 2% glucose (designated as BHI-Mn-G) (Veen & Abee, 2011). An overnight LB culture of *M. morganii* MM190 and the MRS broth cultures of *Lactobacillus* were used to inoculate BHI-Mn-G to an initial optical density of 0.1 at 600 nm. The microplates were incubated in stable conditions for 48 h at 37 °C. To estimate total biofilm biomass, the broth was removed from the wells, and biofilms were washed with distilled water and stained with 0.1% (w/v) crystal violet

(Sigma-Aldrich) for 1 h at room temperature. After vigorous washing with water, the stained biofilms were dissolved in 96% ethanol and biofilm-associated dye was measured in a microplate reader Tecan Infinite F200 PRO as A570 nm (Yarullina *et al*., 2013).

Since crystal violet staining alone is not adequate to assess antagonism in multi-species biofilms, we enumerated culturable biofilm-embedded and planktonic bacteria using the drop plate assay (Herigstad *et al*., 2001). To detach biofilm-embedded cells, the wells were washed twice with 0.9% NaCl to remove the non-adherent cells, and the biofilms were suspended in 0.9% NaCl by scratching the well bottoms. 10-fold dilution series of culture liquid and the suspended biofilm were prepared and dropped by 3 μL-drops onto MRS agar to cultivate lactobacilli and BHI agar (BD Bacto) for M. morganii. CFUs were counted from the two last drops containing countable amount of colonies (5–15 colonies) and averaged.

Statistical analysis

All experiments were performed in triplicate. Quantitative data are presented as the means ± standard deviations that were analyzed using GraphPad Prism 5. Statistical differences between mean values were determined using Student's t test at a significance level of $P < 0.05$.

Results

Strains identification

Based on MALDI Biotyper analysis, six strains were assigned to *Limosilactobacillus fermentum* species, by two strains – to *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus*, and *Ligilactobacillus salivarius*. For the strain FCa8L the species identification was considered as probable since the log score was < 2 (Table 1).

Tolerances to acid and bile

The ability to survive under gastric conditions including low pH and bile presence is an important property of lactobacilli strains used as probiotics and in the food industry. It is strongly species- and strain-dependent. We investigated the tolerance of the isolates by exposing cells to the simulated gastric conditions for 2 h. Four human intestinal isolates and strain FCa3L demonstrated remarkable resistance with no viability reduction. Fecal isolates were the most susceptible to acid and bile of all tested strains (Table 1).

Adhesion capacity

Adhesion capacity was evaluated in hydrophobicity and autoaggregation assays (Table 1). The surface hydrophobicity of lactobacilli cells measured by the MATS method ranged from (4.82 ± 0.35) % to (28.46 ± 2.00) %. According to the ranking offered by (Li & McLandsborough, 1999), all the strains were considered moderately hydrophilic (10–29%) except for the strains LS-4.4 and HF-D1, which were strongly hydrophilic (<10%). Autoaggregation ability after 4 h of incubation varied from (30.4 ± 6.5) % to (58.7 ± 7.9) %. After 24 h of incubation increase in the autoaggregation varied in a wide range from 7.7% (strain LR-1) to 100.6% (strain FCa1L). No relation was observed between hydrophobicity and autoaggregation.

Antibiotic resistance

All the strains were susceptible or moderately susceptible to ampicillin, chloramphenicol, erythromycin, tetracycline, rifampicin, and clindamycin (Table 2). Most of the strains were resistant to vancomycin, ciprofloxacin, and aminoglycosides (amikacin, kanamycin, and gentamicin). Resistance to the aminoglycoside antibiotic streptomycin varied among tested strains: six strains demonstrated resistance, five strains were susceptible, and HF-F3 was moderately susceptible. Human intestinal isolates, especially LR-1, exhibited unusual for lactobacilli phenotypic antibiotic sensitivity pattern, presumably, resulted from previous antibiotic therapy. Overall, the tolerance to aminoglycosides, vancomycin, and ciprofloxacin revealed in the tested strains is likely to be intrinsic, chromosomally encoded and not transferable in lactobacilli.

Table 1

Probiotic properties of lactobacilli

No.	Strain	Log score*	Source	Survival rate, %	Hydrophobicity, %	Autoaggregation, %		Acidification rate		
						4 h	24 h	ΔpH	Ia	TTA
1	*Lacticaseibacillus rhamnosus* LR-1	2.061	Colonic samples obtained from the patients, who undergone colorectal surgery in the Republican Clinical Hospital of the Ministry of Health of the Republic of Tatarstan in September - December 2015	96.02 ± 10.16	19.51 ± 0.38	42.7 ± 4.3	46.0 ± 3.9	2.53 ± 0.22	0.39 ± 0.11	1.12 ± 0.12
2	*Lacticaseibacillus rhamnosus* LR-2.7	2.151		93.40 ± 10.56	16.34 ± 1.08	46.1 ± 5.4	68.8 ± 1.0	2.74 ± 0.35	0.31 ± 0.01	1.07 ± 0.17
3	*Ligilactobacillus salivarius* LS-2.1	2.172		100.97 ± 19.62	15.66 ± 0.76	53.4 ± 2.3	80.2 ± 5.7	2.74 ± 0.34	0.33 ± 0.05	1.48 ± 0.19
4	*Ligilactobacillus salivarius* LS-4.4	2.123		89.24 ± 11.02	7.15 ± 0.62	41.2 ± 6.4	77.8 ± 1.3	2.87 ± 0.06	0.39 ± 0.08	1.42 ± 0.18
5	*Limosilactobacillus fermentum* HF-A4	2.083	Fecal sample from healthy individual, male, age 20	10.81 ± 1.08	23.12 ± 1.97	37.1 ± 1.1	55.1 ± 7.0	2.72 ± 0.33	0.30 ± 0.04	0.55 ± 0.08
6	*Limosilactobacillus fermentum* HF-C1	2.052	Fecal sample from healthy individual, male, age 26	9.72 ± 3.87	10.94 ± 1.50	30.4 ± 6.5	48.5 ± 2.9	2.25 ± 0.32	0.32 ± 0.03	0.93 ± 0.03
7	*Limosilactobacillus fermentum* HF-D1	2.007	Fecal sample from healthy individual, female, age 27	17.50 ± 6.48	4.82 ± 0.35	47.9 ± 2.4	64.9 ± 4.9	2.73 ± 0.33	0.35 ± 0.05	1.37 ± 0.22
8	*Limosilactobacillus fermentum* HF-E1	2.093	Fecal sample from healthy individual, male, age 35	7.95 ± 0.85	22.40 ± 3.67	48.4 ± 1.0	76.0 ± 2.1	2.72 ± 0.36	0.30 ± 0.06	1.50 ± 0.24
9	*Limosilactobacillus fermentum* HF-F3	2.073	Fecal sample from healthy individual, female, age 26	10.09 ± 7.43	28.46 ± 2.00	38.8 ± 2.0	68.5 ± 3.5	2.18 ± 0.35	0.28 ± 0.05	0.97 ± 0.02
10	*Lactiplantibacillus plantarum* FCa1L	2.194	Sauerkraut collected from a local market (Kazan, Republic of Tatarstan, Russia)	33.81 ± 9.87	14.37 ± 0.88	31.4 ± 3.7	63.0 ± 6.9	2.88 ± 0.10	0.32 ± 0.01	1.54 ± 0.24
11	*Lactiplantibacillus plantarum* FCa3L	2.172		115.94 ± 7.37	21.92 ± 1.59	58.7 ± 7.9	67.2 ± 3.7	2.63 ± 0.38	0.33 ± 0.02	1.46 ± 0.24
12	*Limosilactobacillus fermentum* FCa8L	1.893		66.35 ± 8.72	20.68 ± 1.50	43.1 ± 7.5	66.3 ± 6.0	2.90 ± 0.09	0.37 ± 0.02	1.04 ± 0.09

Note: * Log score – identification score given by MALDI Biotyper (reliable species identification: ≥ 2.0; secure genus and probable species identification: 1.7-2.0).

Antibiotic resistance

No.	Strain	Antibiotics *				
		Ampicillin	**Amikacin**	**Chloramphenicol**	**Ciprofloxacin**	**Clindamycin**
1	*L. rhamnosus* LR-1	S (18 ± 2.8)	S (18 ± 2.8)	I (17 ± 1.4)	R (5 ± 0)	S (18 ± 0)
2	*L. rhamnosus* LR-2.7	S (28 ± 2.8)	R (14 ± 0)	S (28 ± 2.8)	R (5 ± 0)	S (27 ± 4.2)
3	*L. salivarius* LS-2.1	S (32 ± 0)	R (14.5 ± 0.7)	S (29 ± 1.4)	S (20 ± 0)	S (14.5 ± 0.7)
4	*L. salivarius* LS-4.4	S (38 ± 2.8)	R (10 ± 0)	S (30 ± 0)	S (23 ± 1.4)	S (25 ± 1.4)
5	*L. fermentum* HF-A4	S (26.5 ± 0.7)	R (7.0 ±1.4)	S (22 ± 2.8)	R (6 ± 0)	S (16 ± 1.4)
6	*L.fermentum* HF-C1	S (26.5 ± 2.1)	R (6.5 ± 0.7)	S (23.5 ± 0.7)	R (7 ± 1.4)	S (18 ± 1.4)
7	*L.fermentum* HF-D1	S (31.5 ± 0.7)	R (10 ± 1.4)	S (22.5 ± 2.1)	R (6 ± 0)	S (13 ± 1.4)
8	*L. fermentum* HF-E1	S (31 ± 1.4)	R (7.5 ± 0.7)	S (25 ± 1.4)	R (7.5 ± 2.1)	S (17.5 ± 2.1)
9	*L. fermentum* HF-F3	S (32.5 ± 0.7)	R (6 ± 0)	S (26 ± 1.4)	R (6 ± 0)	S (15.5 ± 0.7)
10	*L. plantarum* FCa1L	S (32.5 ± 3.5)	R (5 ± 0)	S (28 ± 2.8)	R (5.5 ± 0.7)	S (14.5 ± 0.7)
11	*L. plantarum* FCa3L	S (25 ± 3.5)	R (5 ± 0)	S (28 ± 2.8)	R (6 ± 0)	S (12.5 ± 3.5)
12	*L. fermentum* FCa8L	S (31.5 ± 2.1)	R (7 ± 0)	S (24.5 ± 2.1)	R (10.5 ± 0.7)	S (14.5 ± 0.7)

Table 2

of lactobacilli

Antibiotics *						
Erythromycin	**Gentamicin**	**Kanamycin**	**Rifampicin**	**Streptomycin**	**Tetracycline**	**Vancomycin**
S (23 ± 1.4)	S (19 ± 1.4)	I (17 ± 1.4)	S (19 ± 1.4)	S (25.5 ± 0.7)	S (19 ± 1.4)	S (20 ± 0)
S (26 ± 0)	S (14 ± 0.0)	S (19.5 ± 0.7)	S (24 ± 0)	S (19 ± 1.4)	S (29 ± 1.4)	R (10 ± 0.7)
S (30 ± 0)	R (10 ± 0)	R (12 ± 0)	S (20 ± 0)	S (19.5 ± 0.7)	S (32 ± 0)	S (24 ± 0)
S (29 ± 1.4)	R (12 ± 0)	I (16 ± 0)	S (25 ± 1.4)	S (16 ± 0)	S (33 ± 1.4)	S (24 ± 0)
S (21 ± 1.4)	R (10 ± 1.4)	R (8.5 ± 2.1)	S (22 ± 0)	R (7.5 ± 0.7)	I (15.5 ± 0.7)	R (6 ± 0)
S (20 ± 1.4)	R (8.5 ± 2.1)	R (8.5 ± 0.7)	S (24 ± 1.4)	R (6.5 ± 0.7)	S (24.5 ± 2.1)	R (7 ± 1.4)
S (20 ± 1.4)	R (10 ± 1.4)	R (7 ± 1.4)	S (23.5 ± 2.1)	R (6 ± 0)	S (19 ± 1.4)	R (7 ± 1.4)
S (20.5 ± 0.7)	R (9.5 ± 0.7)	R (7.5 ± 2.1)	S (22 ± 0)	R (7 ± 1.4)	I (16.5 ± 0.7)	R (6 ± 0)
S (19.5 ± 0.7)	R (6 ± 0)	R (6 ± 0)	S (23 ± 1.4)	I (11.5 ± 0.7)	S (23.5 ± 0.7)	R (7 ± 1.4)
S (22 ± 1.4)	R (5 ± 0)	R (5 ± 0)	S (25.5 ± 2.1)	R (5 ± 0)	S (23.5 ± 2.1)	R (5 ± 0)
S (20.5 ± 1.4)	R (5.5 ± 0.7)	R (6 ± 0)	S (21 ± 0)	R (5.5 ± 0.7)	S (19 ± 1.4)	R (5 ± 0)
S (20.5 ± 0.7)	R (6 ± 1.4)	R (5 ± 0)	S (20.5 ± 0.7)	S (22.5 ± 3.5)	S (21 ± 0)	R (9.5 ± 0.7)

Note: * Diameters of inhibition zones (mm) were interpreted as susceptible (S), intermediate (I), or resistant (R) according to (Melo *et al.*, 2017).

Acidification activity

Table 1 shows the results of the acidity measurement in the MRS medium after the lactobacilli growth for 48 h. During incubation, all the *Lactobacillus* strains decreased the pH, associated with an increase in total titratable acidity (TTA). The values of ΔpH were almost similar for all the tested strains ranging between (2.18 ± ± 0.35) and (2.90 ± 0.09). Similarly, I_a values were also similar and fell within the range between (0.28 ± 0.05) and (0.39 ± 0.11), indicating similar growth of the tested lactobacilli. Meanwhile, titratable acidity varied widely among the lactobacilli, with the highest level being demonstrated by FCa1L (1.54 ± ± 0.24) and the lowest level of HF-A4 (0.55 ± ± 0.08). Although TTA and pH are both measures of acidity, no correlations between TTA values and pH changes (ΔpH and Ia) were observed.

Antagonistic activity of lactobacilli against Morganella morganii

Table 3 displays the results of the agar spot test. The anti-Morganella activity was found to be a strain-dependent phenomenon for both *Lactobacillus* and *M. morganii*. As notable by the presence of significant inhibition halos around the colonies of lactobacilli, *M. morganii* strains MM1 and MM4 were sensitive to the antagonistic activity of only seven and six *Lactobacillus* isolates, respectively. With the exception of HF-A4, which completely lacked the anti-Morganella activity, all the isolates demonstrated antagonistic activity against *M. morganii* MM190 with zones of inhibition from 7.0 to 8.5 mm (Fig. 1).

Further we examined whether the inhibitory effect of the *Lactobacillus* strains on the *M. morganii* growth was associated with the production of bacteriocins. In the agar spot test performed to detect bacteriocins all the *Lactobacillus* strains were unable to inhibit the growth of *M. morganii* MM190. Further, using the crude bacteriocin preparations (CBP) from stationary phase cultures, we observed that with the exception of LR-1, CBP of all the *Lactobacillus* strains used in this study showed anti-Morganella activity, notable by the presence of inhibition halos on the lawn of *M. morganii* MM190.

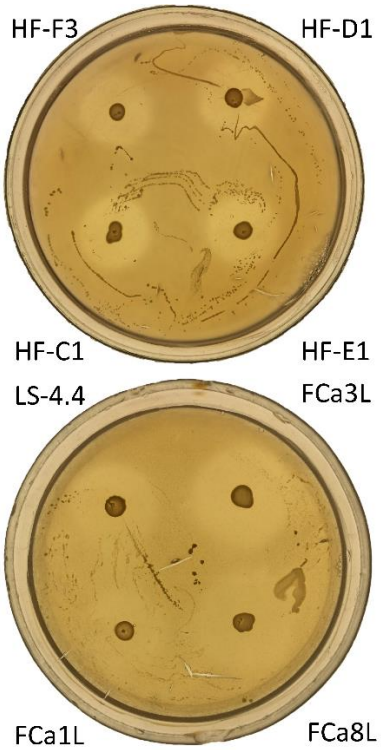

Fig. 1. Inhibition zones of lactobacilli strains (grown on MRS agar plates as spot forms) against *M. morganii* MM190 (grown in the overlay semi-solid LB medium)

All tested *Lactobacillus* strains displayed co-aggregation properties with *M. morganii* MM190, ranging from (1.8 ± 1.5) % for HF-D1 to (29.2 ± 2.5) % for LS-4.4. Regarding the autoaggregation ability of *M. morganii* MM190, it showed percentage (54.9 ± 1.9) after 4 h of incubation and (82.2 ± 6.6) after 24 h of incubation.

We chose five *Lactobacillus* strains (LS-2.1, LS-4.4, HF-A4, HF-D1, and FCa8L) to study their ability to eradicate *M. morganii* in the mixed-species biofilm. Lactobacilli and *M. morganii* MM190 were inoculated together in the BHI-Mn-G broth at equal cell densities and incubated for 48 h with subsequent CFU counts of the enterobacterium and lactobacilli in the culture liquid (Fig. 2B) and in the residual biofilm (Fig. 2C). The data obtained from the crystal violet assay (Fig. 2A) and CFU counts (Fig. 2B, C) revealed that *M. morganii* MM190 is able to form mixed-species biofilms with all the tested *Lactobacillus* strains. In the mixed-species biofilms both lactobacilli and *M. morganii*

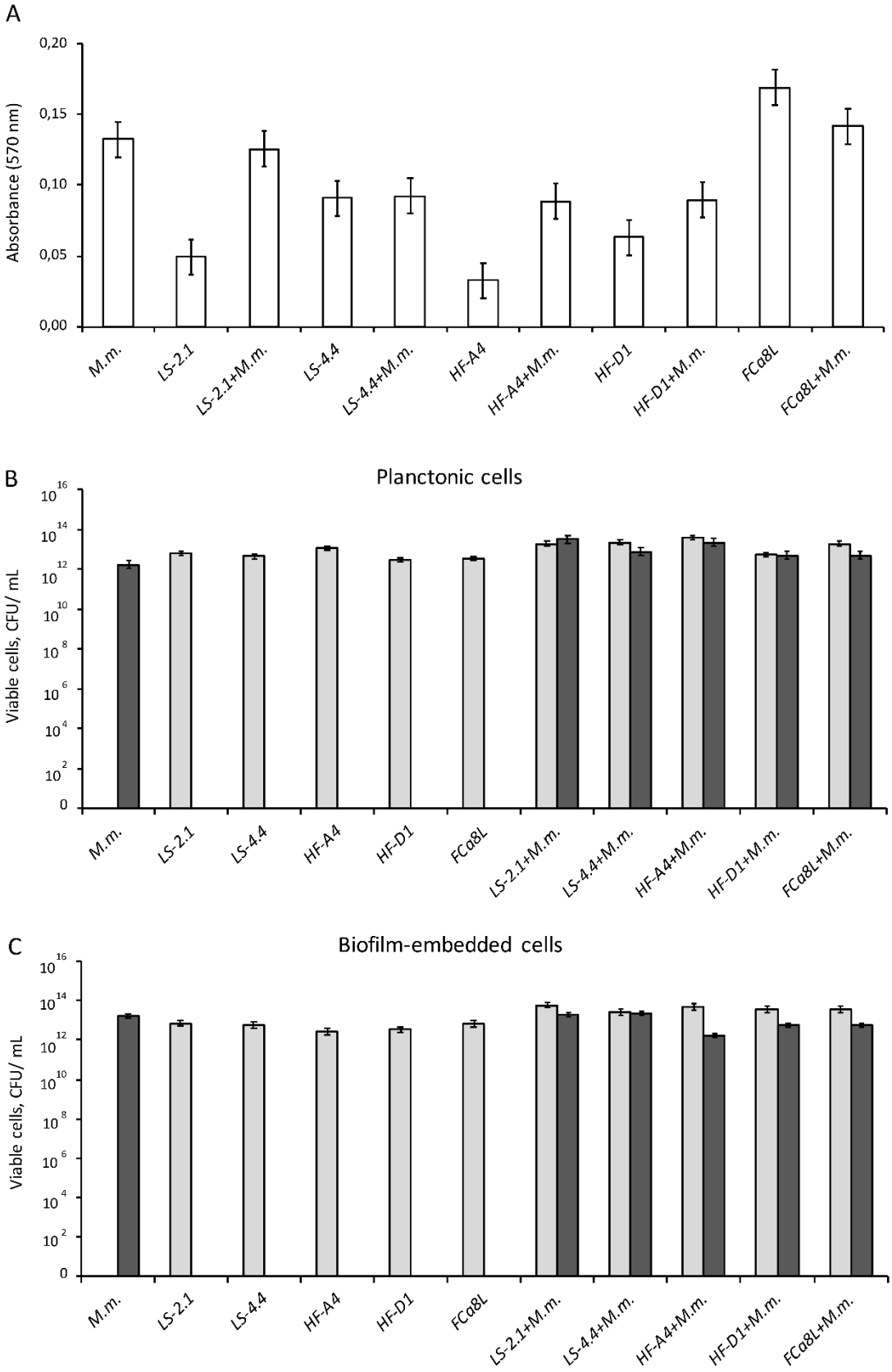

Fig. 2. Quantification of 48 h mixed-species biofilms of *M. morganii* MM190 and lactobacilli strains using (A) the crystal violet assay, (B) planktonic cell counts, and (C) biofilm-embedded cell counts

Table 3

Antagonistic activity of lactobacilli against *Morganella morganii*

No.	Strain	Growth inhibition, mm			Bacteriocins production	Coaggregation with *M. morganii* MM190 after 4 h, %
		M. morganii MM1	*M. morganii* MM4	*M. morganii* MM190		
1	*L. rhamnosus* LR-1	6.5 ± 2.1	0.5 ± 0.7	8.0 ± 0.0	–	5.5 ± 3.1
2	*L. rhamnosus* LR-2.7	0	0	8.5 ± 0.7	+	16.4 ± 6.4
3	*L. salivarius* LS-2.1	1.0 ± 1.4	1.0 ± 1.4	8.5 ± 0.5	+	20.7 ± 0.6
4	*L. salivarius* LS-4.4	7.5 ± 0.7	0	8.5 ± 0.0	+	29.2 ± 2.5
5	*L. fermentum* HF-A4	0.5 ± 0.7	0	0	+	20.1 ± 5.1
6	*L.fermentum* HF-C1	0	0	7.5 ± 0.0	+	10.7 ± 4.2
7	*L.fermentum* HF-D1	5.5 ± 0.7	5.0 ± 0.8	7.0 ± 0.7	+	1.8 ± 1.5
8	*L. fermentum* HF-E1	7.0 ± 1.4	5.5 ± 1.9	7.5 ± 0.5	+	16.1 ± 4.3
9	*L. fermentum* HF-F3	6.0 ± 2.8	6.0 ± 1.0	7.5 ± 0.5	+	27.7 ± 0.1
10	*L. plantarum* FCa1L	6.5 ± 0.7	6.3 ± 0.6	8.0 ± 0.5	+	22.0 ± 1.7
11	*L. plantarum* FCa3L	1.5 ± 2.1	6.5 ± 0.7	8.5 ± 0.0	+	18.6 ± 2.0
12	*L. fermentum* FCa8L	8.0 ± 0.0	6.3 ± 1.7	8.5 ± 0.7	+	5.0 ± 0.6

MM190 reached 12–13 log10 CFU/mL after 48 h of incubation, the same cell densities as in the single species biofilms. That is, all the tested *Lactobacillus* strains were unable to repress the pathogens' growth in both culture liquid and biofilms.

Discussion

In this study, we isolated 12 lactobacilli strains and characterized their beneficial properties with regard to their antagonistic activity against multi-resistant and virulent pathogen *M. morganii*. Growing antibiotic resistance of *M. morganii* complicates treatment of its infections and increases the importance of the search for probiotic strains which are able to replace antibiotics in anti-Morganella therapy. Besides antimicrobial activity these promising strains must be resistant to the challenges met in the GIT. Tolerance to the hostile gastrointestinal environment, adherence to the intestinal mucus and antibiotic resistance represent important prerequisites for a probiotic to be of benefit to human health (Saarela *et al*., 2010). These properties are strain-specific and thus need to be evaluated for every new promising probiotic isolate.

In this study, harsh stress factors of the GIT, which include digestive enzymes, acidity, and biliary salts, were mimicked by the synthetic gastric fluid (pH 2.5) (Beumer *et al*., 1992). Five lactobacilli strains, namely LR-1.1, LR-2.7, LS-2.1, LS-4.4, and FCa3L, exhibited good survival rate over the 2 h of exposure to simulated gastric juice, while the poorest survivor was HF-E1 (Table 1).

Bacterial adhesion and autoaggregation (ability to form floccules) are key factors for colonization of mucosa in the gastrointestinal and urogenital tracts (Kos *et al*., 2003). All *Lactobacillus* strains tested in this study showed percentage of autoaggregation higher than 30% after 4 h of incubation, with the highest level being demonstrated by FCa3L (58.7 ± 7.9 %), followed by LS-2.1 (53.4 ± 2.3 %) (Table 1). After 24 h of incubation the highest level of autoaggregation was exhibited by LS-2.1 (80.2 ± ± 5.7 %). The autoaggregation capacity of lactobacilli usually ranges from low to moderate (Bouchard et al., 2015, Tuo *et al*., 2013). The standard probiotic cultures like *L. johnsonii* LA1, *L. acidophilus* LA7, and *L. rhamnosus* GG after 5 h of incubation self-aggregated at the level of 40.4 ± 0.4%, 46.5 ± 2.0%, and 41.39 ± 3.30%, respectively (Kaushik *et al*., 2009, Tuo *et al*., 2013). Bacteria with the ability to autoaggregate remain in the intestines for a longer time and thus better exert their probiotic

effects (Kemgang *et al*., 2014). In this study cell surface hydrophobicity was used as the measure of adhesive ability. It is known, that hydrophobicity is in direct ratio to the adhesive ability (Van Loosdrecht *et al*., 1987). All tested strains revealed hydrophilic cell surface and thus low potential to adhere to the intestinal mucus. Probiotic strains *L. acidophilus* M92 and L. rhamnosus GG demonstrated hydrophobic cell surfaces (Kos *et al*., 2003; Deepika *et al*., 2009). However, another well-known probiotic strain *L. plantarum* 8PA3 showed hydrophilic properties (23.2 ± 1.03 %) (Gavrilova *et al*., 2019). High hydrophobicity usually results from the presence of (glyco)proteinaceous compounds at the cell surface, whereas hydrophilic surfaces are associated with the presence of polysaccharides (Kos *et al*., 2003; Deepika *et al*., 2009).

Characterization of the antibiotic resistance is relevant with regard to two aspects: the risk of horizontal transfer of resistance genes from lactobacilli and combination of probiotic bacteria with antibiotic treatment (Anisimova & Yarullina, 2018). *Lactobacillus* strains exhibited susceptibility to the majority of the antibiotics, with the exception of vancomycin, ciprofloxacin, and aminoglycosides (Table 2). Such a profile was especially clearly displayed in six isolates: HF-A4, HF-C1, HF-D1, HF-E1, FCa1L, and FCa3L. It is considered typical for lactobacilli and safe regarding the spread of the antibiotic resistance genes in the environment since resistances to vancomycin, ciprofloxacin, and aminoglycosides are intrinsic for *Lactobacillus* spp. (Gueimonde *et al*., 2013). Besides, resistance profiles of these isolates support the possibility of their combination with different classes of antibiotics that target cell wall, protein or nucleic acid synthesis.

Characterization of the inhibitory potential of *Lactobacillus* against pathogenic bacteria *M. morganii* was the main focus of our study. Screening for the isolates with anti-Morganella activity revealed five strains (namely HF-D1, HF-E1, HF-F3, FCa1L, and FCa8L) which inhibited the growth of all three *M. morganii* strains and one strain (HF-A4), which completely lacked the anti-Morganella activity (Table 3). *M. morganii* MM190 was the most sensitive strain to the inhibitory activity of lactobacilli. Thus, antagonism between *Lactobacillus* and *M. morganii* was strain-specific for both sides of bacterial interaction.

In search for the factors involved into the antagonistic effect of lactobacilli, we demonstrated that 11 tested *Lactobacillus* strains had the ability to produce bacteriocin active against *M. morganii*, but its concentration appeared to be not enough to exhibit its growth inhibitory activity in the culture. The nutrient medium and incubation conditions have strong influence on the production of bacteriocins by lactic acid bacteria (Garsa *et al*., 2014). Further studies will optimize the conditions for high production of bacteriocins from the producer strains first described in this work.

Also, antagonistic effects of the *Lactobacillus* strains against *M. morganii* were not correlated with any tested measure of acidity. Nevertheless, HF-A4 which almost did not affect *M. morganii* growth was the weakest producer of organic acids as determined by the lowest level of TTA among the tested strains. Still, HF-A4 decreased the pH to a similar level as the strains HF-D1, HF-F3, FCa1L, and FCa8L, which exhibited the highest antagonistic activity against *M. morganii*. Thus, our results demonstrated the ability of the lactobacilli to inhibit the growth of *M. morganii*. The ground of the anti-Morganella activity of lactobacilli remains obscure. Our results did not show the association of antagonistic activity with bacteriocins production and pH decrease via production of organic acids. Yet, they do not exclude the impact of these factors on the discovered antagonistic effect.

All the isolates exhibited some degree of coaggregation with *M. morganii* MM190, with the highest level being demonstrated by LS-4.4 (29.2 ± 2.5 %), followed by HF-F3 (27.7 ± ± 0.1%) (Table 3). Coaggregation helps probiotics to combat pathogens in several ways. First, due to coaggregation probiotic strains may produce antimicrobial substances in very close vicinity to the pathogen cells (Reid *et al*., 1988). Second, via coaggregation lactobacilli may form a barrier that prevent colonization by

pathogenic bacteria (Ferreira *et al*., 2011). Besides, auto- and coaggregation play a critical role in the formation of bacterial multi-species biofilms over the host tissues (Rickard *et al*., 2003).

To study the antagonism of the *Lactobacillus* strains against *M. morganii* cells embedded into the biofilm we used five *Lactobacillus* strains with different probiotic and antagonistic properties, namely LS-2.1, LS-4.4, HF-A4, HF-D1, and FCa8L. Mixed-species biofilms give the opportunity to assess the influence of one microorganism on the growth of another when both are incubated together (Gavrilova *et al*., 2019). We observed that none of the *Lactobacillus* strains was able to reduce significantly the microbial counts of planktonic and biofilm-embedded *M. morganii* MM190 cells when compared to *M. morganii* MM190 growing alone (Fig. 2B, C).

Conclusion

In the present work we described novel *Lactobacillus* strains capable to inhibit the growth of the clinical isolates of *M. morganii* in vitro. Estimated probiotic properties, such as acid tolerance, adhesion, autoaggregation, acidification rate, and antagonistic activity were strain-specific and irrespective of the origin or taxonomic group. *L. plantarum* FCa3L isolated from sauerkraut showed strong potential as a probiotic for application in anti-Morganella therapy because it exhibited strong survival ability under conditions that mimic gastrointestinal transit, high acidification rate, sufficient percentage of autoaggregation and coaggregation with *M. morganii*, bacteriocin production, and typical antibiotic resistance profile evidently without potentially transferable resistance determinants.

Acknowledgments

This work was funded by Russian Science Foundation (grant N 22-16-00040) and by the Kazan Federal University Strategic Academic Leadership Program (PRIORITY-2030).

Conflict of interest: the authors declare that they have no conflict of interest.

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