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RESEARCH ARTICLE

ASSESSMENT OF PHYSIOLOGICAL PROPERTIES OF SOME LACTIC ACID BACTERIA ISOLATED FROM THE INTESTINE OF CHICKENS USE AS PROBIOTICS AND ANTIMICROBIAL AGENTS AGAINST ENTEROPATHOGENIC BACTERIA

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Abstract

The aim of the present study was to isolate lactic acid bacteria able to produce antibacterial compounds from healthy chicken intestine against enteropathogenic bacteria. Isolates were characterized morphologically and biochemical tests were carried out for identification. The agar diffusion method was used to evaluate the antimicrobial activities. Growth with bile salts and resistance to acidic pH were tested in broth medium. Susceptibility to antibiotics was also tested. Fifteen strains were isolated and characterized. All the strains proved to tolerate bile salts at concentration of 0.4% and only three strains were resistant to pH 3.0. The antimicrobial test showed that all the lactic acid bacteria isolates provide an antimicrobial activity against *Enterococcus faecalis, Escherichia coli, Salmonella arizonae, Enterococcus avium* and *Enterococcus casseliflavus.* The isolates were resistant to trimethoprim, cefixim and erythromycin and susceptible to chloramphenicol, amoxicillin/clavulanic acid and tetracyclin. Among all the isolates, three strains *Enterococcus faecium* 1LC, *Lactococcus cremoris* NPL and *Pediococcus* spp. L4 were selected for several properties, tolerance to acid, bile salts and susceptibility to antibiotics. Selected strains were the most promising that may be useful as probiotic adjunct in poultry.

Keywords: Lactic acid bacteria, chicken intestine microbiota, antimicrobial activity

Introduction

Poultry production has become an important sector of economy for farmers. This has contributed to increasing use of antibiotics as health protective factor, which has been shown to have negative effect not only to human but also to animal. It is well recognized that the intensive use of antibiotics contributes to the development of antibiotic resistance both in humans and animals. Antibiotics are now forbidden in animal feeding in the European Union countries. In this respect, the development of alternatives that will permit the respect of law without reducing their competitiveness is being intensified. Many studies

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have focused on antimicrobial activity release of many compounds produced by lactic acid bacteria (LAB) to prevent the growth of pathogenic bacteria such as *Salmonella enterica and Escherichia coli* (Ashraf *et al.*, 2005). Before using bacterial strains as probiotics, they must be resistant to certain conditions of the intestinal tract. Most bacteria do not survive well at low pH values. The severe acidic conditions of the proventiculus and gizzard could have an adverse effect on the bacteria. Thus, it was suggested that microbial cultures to be used as probiotics should be screened for their resistance to acidic pH (Conway *et al.*, 1987). Once the bacteria reach the intestinal tract, their ability to survive depends on their resistance to bile and low pH (Gilliland *et al.*, 1984). The knowledge of antibiotic susceptibilities of potential probiotic strains is also necessary (Charteris *et al.*, 1998).

The objective of this study was to physiological an biochemical characterize of some lactic acid bacteria strain isolated from the crop and intestinal contents of poultry for their potential application as a probiotic feed supplement based on resistance in conditions of the intestinal tract and antimicrobial activity.

Materials and methods

Bacterial strains

Lactic acid bacteria were isolated from chicken crop and intestine of poultry birds at the Laboratory of Microbiology of University Institute of Technology and National Advanced School of Agro-industrial Sciences, the University of Ngaoundere, Cameroon. The isolation was performed by the routine microbiological isolation procedure and inoculation was performed on a selective medium made up of MRS agar plates. Ten fold dilutions of crop and intestinal isolates were spread on the surface of the medium. Colonies were selected from high dilution (5 log CFU) in MRS agar plates. Strains were grown in the MRS broth at 37°C, for 18 hours.

As indicator strains were used *Enterococcus faecalis, Escherichia coli, Salmonella arizonae, Enterococcus avium* and *Enterococcus casseli-flavus* from Laboratory of Microbiology, of the University Institute of Technology and National Advanced School of Agro-industrial Sciences, University of Ngaoundere, Cameroon

Physiological and biochemical tests of isolated LAB

All the strains under examination were subcultured twice overnight in MRS broth and were initially tested for Gram stain affinity, motility, catalase and oxidase production. Cells morphology and colonial characteristics on MRS agar were also examined. Only Gram positive, catalase-negative and oxidase-negative isolates were selected. Growth at different temperatures was measured in MRS broth after incubating for 18 hours at 10°C and 45°C. Growth in the presence of 6.5% NaCl was performed in MRS broth for 18 hours. The strains were characterized and classified by using API Strips (BioMerieux, Marcy-l'Etiole, France) and other complementary tests according to the criteria of Bergey's Manual of Determinative Bacteriology (Bergey *et al.*, 1989).

Antibacterial activity assays

The antibacterial activity spectrums of cells free supernatants of different lactic acid bacteria were determined using the agar diffusion method (Tagg and McGiven, 1971) against indicator bacterial strains. The supernatant from 24 hours culture of LAB were filtrated with 0.20µm pore size syringe. Aliquot of 50µl of sterile supernatant were placed in 6mm diameters well that has been cut in Mueller Hinton agar plates and spotted on with the pathogenic bacteria. After 18-24 hours of incubation, the diameters of the zones of growth inhibition were measured. The screening of the antibacterial substances was performed by using the agar spot test and the well diffusion method, as described by Barefoot and Klaenhammer (1983) and Tagg and McGiven (1971) respectively.

Lactic acid bacteria resistance in conditions of the intestinal tract evaluation

Acid tolerance

Lactic strains were grown in MRS broth at 37°C for 18 to 24 hours. Biomass was recovered by centrifugation at 7 500 g for 15 min at 4°C and washed twice in sterile saline phosphate buffered

(PBS, 0.1M phosphate buffer with 0.8% NaCl, pH 7.0). Cells were inoculated in MRS broth at pH 2.0 and 3.0 and incubated at 37°C for 18hours. Samples were taken after 2, 3 and 24 hours of incubation occurred in fresh MRS broth to assess the resistance of lactic acid bacteria at different pH values. The increase of turbidity in the fresh broth therefore reflects bacterial growth which means that the bacteria resisted the acidic conditions and the optical density was monitored at 600 nm after 3 and 24 hours. Control sample without acidification were also prepared and similarly handled. The test was performed in triplicates.

Bile tolerance

The method used for testing bile tolerance was similar to that described by Gilliland *et al.* (1984). The cells were grown overnight in MRS broth and then were inoculated into tubes containing MRS broth with 0.4% bile salts (Sigma) and MRS broth without bile used as control. The optical density was monitored at 600 nm after 4 and 24 hours of incubation against non-inoculated blank (Gotcheva *et al.*, 2002). Each lactic acid bacteria strain cultured in MRS broth at optimal pH (6.5) was runned as controls. All tests were carried out in triplicate.

Resistance to antibiotics

The antibiotic resistance was evaluated using the agar plate method according to Charteris *et al.*, (1998). This method is a modification of the agar overlay diffusion method of the National Committee for Clinical Laboratory Standards. Susceptibility testing was studied in triplicates. Man Rogosa Sharp agar (Difco) was used as a basal medium for bacterial growth. Culture suspensions were obtained after incubation at 37°C in MRS broth and spread on agar plates at 0.5 McFarland. For the agar spot test, 4µl of LAB isolates were spotted on the surface of MRS agar medium. Plates were kept at room temperature for 1h. Multipositional dispenser (BIO-RAD, France) applied standard discs and incubated anaerobically at 37°C for 24hours. The diameter of the inhibition zones were measured and then compared with the values of susceptibility interpretative break points issued by the National Committee for clinical Laboratory Standards (NCCLS, 1999) and was expressed in terms of resistance (R), moderate susceptibility (MS), and susceptibility (S).

Statistical analysis

Data were statistically analysed using one way analysis of variance (ANOVA) test for significance at $p \leq 0.05$ using analyze it software version 1.73

Results and discussions

Characterization and identification of the selected lactic bacteria

Lactic acid bacteria strains able to produce antibacterial compounds were isolated from chicken crop and intestinal contents. A number of bacterial species were characterized as Gram positive, catalase-negative and oxidase negative and non spore forming bacteria. The isolates were furthermore identified asbelong of genus *Enterococcus, Lactococcus* and *Pediococcus,* coded according to data shown in Table 1. The morphological characteristics, according to Bergey's manual of Determinative of Bacteriology and biochemical properties showed the similarity in characteristics with *Enterococcus faecium, Lactococcus cremoris, Pediococcus* spp. (Table 2 and Table 3).

Table 1. *Morphological and physiological characteristics of the lactic acid bacteria isolated strains*

Strain of LAB	Gram staining	Shape	pH 9.6	6. 5% NaCl	Growth at 10°C	Growth at 45°C	Gaz production	Genus
L4	+	coccid	+	+	-	-	-	*Pediococcus*
1LC	+	coccid	-	-	+	+	-	*Enterococcus*
NPL	+	coccid	-	+	+	-	-	*Lactococcus*

+ positive reaction, - negative reaction

Table 2. *Biochemical characteristics of the lactic acid bacteria isolates from chicken intestine on API 50CHL*

	***Pediococcus* L4**
L-arabinose	positive
Ribose	positive
D-xylose	negative
Galactose	positive
D- Glucose	positive
Sorbitol	positive
D-Mannose	negative
Rhamnose	Nd
α-Methyl-D-Glucoside	Nd
N-Acetyl glucosamine	Nd
Amygdaline	Nd
Salicine	Nd
Cellobiose	Nd
Maltose	negative
Lactose	positive
Esculine	positive
Saccharose	negative
Trehalose	positive
Inuline	Nd
Melezitose	Nd
D-Raffinose	Nd
Amidon	negative
β-Gentibiose	Nd
D-Turanose	Nd
D-Tagatose	Nd
Gluconate	Nd
5-ceto-Gluconate	Nd

Nd (not determined)

Antibacterial activity

Antibacterial activity was tested on different enteropathogenic bacteria isolated from poultry used as indicators. Agar diffusion methods describe by Tagg and McGiveen (1971) was used. The growth inhibition showed a clear zone around the tested colonies. The inhibitory zone varied from 9 mm to 26 mm (Table 4). The main revelation in this study was that strains were active both against Gram positive (*Enterococcus avium, Enterococcus cloacae)*, and Gram negative (*Escherichia coli, Proteus mirabilis,* and *Salmonella arizonae)* bacteria. The inhibitory activity of LAB is mainly due to the accumulation of primary metabolites such as lactic acid and acetic acids, ethanol and carbon dioxide. LAB is also capable to produce antimicrobial compounds such as bacteriocins and other compounds with small molecular mass. The production levels and proportions among these compounds depend on the biochemical properties of the strains used and physical and chemical conditions of growth (Tannock, 2004).

Tolerance to acidic pH

Before reaching intestinal tract, probiotic bacteria must first survive during the transit through the stomach where the pH can be as low as 1.5 to 2.0 (Dunne *et al.*, 2001) and remain viable for 4 hours or more (Ouwehand *et al.*, 1999). The results of acid tolerance showed that all tested lactic acid bacteria strains did not survive an incubation period of 2 h at pH 2.0 (Table 5). At pH 3.0, four isolates were resistant after 2 hours. After 3 hours of incubation, only three strains were resistant to acidic pH. The only strain that survived after 24 hours was *Enterococcus faecium*1LC. Generally,

*Enterococcus faecium*1LC survived acidic conditions better than the rest of the tested LAB. Decrease in survival percentage was observed when the exposure time progressed. These results were comparable to those reported by Jin *et al.*, (1998) that most of bacteria strains isolated from gastro intestinal tract of chickens were tolerant to acidic conditions. The time required for feed to pass through the entire alimentary canal is as short as 2.5 hours (Duke, 1977). Therefore, acid tolerance for bacterial strains in chickens is not as crucial as for those in other animals where the feed passage rate is much slower.

Table 3. *Biochemical characteristics of the lactic acid bacteria isolates from chicken intestine on API 20 STREP*

	***Lactococcus* NPL**	***Enterococcus* 1LC**
Pyruvate	positive	positive
Hippurate	positive	Nd
Esculine	negative	positive
Pyrrolidinyl 2-aphtylamide	Nd	Nd
2naphtylamide	Nd	Nd
6-Bromo-2naphtyl	Nd	Nd
α-D-galactopyranoside	Nd	Nd
Naphtol Asi-Bi	Nd	Nd
Glucuronate	Nd	Nd
2-naphtyl ß-D galacto	negative	Nd
Pyranoside	positive	positive
2-naphtyl phosphate	negative	positive
L-leucine 2-naphtyl amide	positive	positive
Arginine	negative	positive
Ribose	positive	positive
Arabinose	negative	positive
Mannitol	positive	Nd
Sorbitol	negative	Nd
Lactose	positive	negative
Trehalose	negative	Nd
Inulin	Nd	Nd
Raffinose	Nd	Nd
Amidon	negative	negative
Glycogene	Nd	Nd

Nd (not determined)

Table 4. Antimicrobial activity of lactic acid bacteria isolated from chicken intestine on entheropathogenic bacterial strains

Strain of LAB	***Escherichia coli***	***Enterococcus cloacae***	***Proteus mirabilis***	***Salmonella arizonae***	***Enterococcus avium***	***Enterococcus casseliflavus***
Pediococcus L4	15,0±0,0^{a}	11,0±0,0^{b}	20,0±0,0^{c}	14,0±0,0^{b}	13,0±0,0^{ab}	20,5±0,7^{a}
Enterococcus 1LC	13,5±0,4^{b}	12,0±0,0^{a}	22,0±0,0^{b}	15,0±0,0^{a}	12,0±0,0^{b}	13,0±0,0^{c}
Lactococcus NPL	12,0±0,0^{c}	12,0±0,0^{a}	23,5±0,7^{a}	12,5±0,7^{c}	13,5±0,7^{a}	14,0±0,1^{b}

$^{a, b, c}$ Values followed with same later in superscript on the same column are not statistically different at $P < 0.05$

Tolerance to bile salts

Tolerance to bile was considered as a prerequisite for colonization and metabolic activity of bacteria in intestine of the host (Havenaar *et al.*, 1992). Therefore when evaluating the potential use of lactic acid bacteria as effective probiotic, it is generally considered necessary to evaluate their ability to resist to the effects of bile salts and acid (Lee and Salimen, 1995). In this study, bile tolerance of strains was also investigated (Table 6). All strains demonstrated significant changes of absorbance ($p<0.05$) from controls when cultured with 0.4% bile concentration. After 6 and 24 hours of incubation all the isolated strains resisted to bile salts. Resistance to bile salts is of great importance to survival and growth of bacteria in the intestinal tract, and thus, is a prerequisite for bacteria to be used as probiotics (Havenaar *et al.*, 1992). The average bile concentration is around 0.3%, and may range up to an extreme of 2.0% during the first hour of digestion (Gotcheva *et al.*, 2002). Bile resistance of some strains vary a lot among the lactic acid bacteria species and between strains themselves (Xanthopoulos *et al.*, 1997). The effect of bile on the survival of lactobacilli has been investigated by several authors and is thought to be linked to the ability of strains to de-conjugate bile acids (Tannock *et al.*, 1989). Bile resistance of some strains is related to specific enzyme activity-bile salt hydrolase (BSH) which helps to hydrolyze conjugated bile, thus reduce its toxic effect (Du Toit *et al.*, 1998). BSH activity has most often been found in organisms isolated from the intestines or faeces of animals (Tanaka *et al.*, 1999).

Table 5. *Effect of acidic pH on the growth of LAB (%)*

pH conditions	**Time**	***Pediococcus* L4**	***Enterococcus* 1LC**	***Lactococcus* NPL**
2.0	t= 2h	0	0	0
3.0	t=2 h	50	70	60
	t= 3 h	20	45	25
	t= 24 h	0	29	0

Table 6. *Survival rate of the lactic acid bacteria selected strains in MRS broth containing 0.4% bile salts*

LAB strains	Time	
	4h %	24h %
Enterococcus faecium 1LC	60	45
Lactococcus cremoris NPL	45	70
Pediococcus sp. L4	55	52

Antibiotic resistance

Selected strains were assayed for their susceptibility to 11 antibiotics. Results are listed in Table 7. The all selected LAB strains were resistant to trimethoprim, cefixim and erythromycin. All the studied strains were susceptible to chloramphenicol, amoxicillin/ clavulanic acid and tetracyclin. A moderate susceptibility was observed with *Pediococcus* sp.L4on oxolinic acid, *Lactococcus cremoris* NPL on penicillin, *Enterococcus faecium* 1LC on sulfamids and erytromycin. The high intrinsic resistance and susceptibility of isolates to a range

of antibiotics was important. Strains that showed resistance to a specific antibiotic can be given at the time of antibiotic treatment. An important drawback of antibiotic resistance is that transfer of antibiotic resistance genes is possible. Because antibiotic resistant genes are generally carried on plasmids, they can be transferred to other bacteria by means of conjugation. This may result to highly antibiotic resistant enteropathogenic bacteria. So it is important to determine whether antibiotic resistant genes are present on chromosomes or on plasmids.

Table 7. *Antibiotics resistance susceptibility*

Antibiotics	**Concentration**	***Enterococcus faecium* 1LC**	***Lactococcus cremoris* NPL**	***Pediococcus* sp. L4**
Trimethoprin	250µg	R	R	R
Tetracyclin	30µg	S	S	S
Streptomycin	500µg	R	R	R
Penicillin	6µg	R	MS	R
Cefixim	10µg	R	R	R
Streptomycin	10µg	R	R	R
Amoxicillin/clavulanic acid	20/10µg	S	S	S
Oxolinic acid	10µg	R	R	MS
Erythromycin	15UI	MS	R	R
Chloramphenicol	30µg	S	S	S
Sulfonamids	200µg	MS	R	R

R- antibiotic resistant; MS - moderate susceptibility; S: susceptible

Conclusions

The results obtained in this study showed the tolerance of strains, in the conditions of high bile salts concentration and low pH value. From this study, it could be concluded that *Enterococcus faecium* 1LC, *Lactococcus cremoris* NPL, *Pediococcus* spp. L4 were resistant to pH 3.0, 6.5% NaCl and bile concentration (0.4%). The antibacterial compounds produced by these isolates may be useful to control the undesirable microbiota of poultry and may be advantageous to the producing strains for their establishment and competition in the gastrointestinal tract. Further studies will be planned to establish whether these strains will be able to adhere to epithelium cells, to aggregate with food borne pathogens before predicting their use as probiotics in conferring health benefits to the host.

References

Ashraf M., Siddique M., Rahman S.U., Arshao M., Khan A.H. (2005). Effect of various microorganims culture feeding against Salmonella infection in broiler chick. *Journal of Agricltural and Social Sciences.*1 (1), 29-31.

Barefoot S.F., Klaenhammer T.R. (1983). Detection an activity of Lactacin B produced by *Lactobacillus acidophilus*. *Applied and Environmental Microbiology*. 45,1808-1815.

Bergey D.H., Harrison F.C., Breed, R.S., Hammer B.W., Huntoon F.M. (1989). *Bergey's Manual of Determinative Bacteriology*. 9th edn. The William and Wilkins Company. London.

Charteris W.P., Kelly P.M., Morelli L., Collins J.K. (1998). Antibiotics susceptibility of potentially probiotic *Lactobacillus* species. *Journal of Food Protection.* 61(12),1636-1643.

Conway P.L., Gorback S.L., Goldin B.R. (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *Journal of Dairy Sciences*. 70,1-12.

Du Toit M., Franz C., Schillinger U., Warles B., Holzappfel W. **(**1998). Characterization and

selection of probiotic lactobacilli for a preliminary mini pig-feeding trail and their effect on serum cholesterol level, faeces pH and faeces moisture contents. *International Journal of Food Microbiology*. 40, 93-104.

Duke G. E. (1977). Avian digestion. In: G.E. Duke, (ed.): *Physiology of Domestic Animals*. 9th ed. Ithaca and London, Cornell University Press, pp. 313-320

Dunne C., O'Mahony L., Murphy L., Thornton G., Morrissey D., O'Halloran S., Feeney M., Flynn S., Fitzgerald G., Daly C., Kiely B., O'Sullivan G.C., Shanahan F., Collins J.K. (2001). In vitro selection criteria of probiotic bacteria of human origin: correlation with in vivo findings. *American Journal of Clinical Nutrition.* 73 (suppl). 386S–392S.

Gilliland S.E., Staley T.E., Bush L.J. (1984). Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct. *Journal of Dairy Sciences.* 67, 3045-3051.

Gotcheva V., Hristozova E., Hristozova T., Guo M., Roshkova Z., Angelov A. (2002). Assessment of potential probiotic properties of lactic acid bacteria and yeast strains. *Food Biotechnology.* 16, 211-225.

Havenaar R., Brink B.T.,Huis Veld J.H.J. (1992). Selection of strains for probiotic use. In: Fuller, R. (ed.) *Probiotics, the Scientific Basis*. Chapman and Hall, London, 1, pp. 209–224.

Jin L.Z., Ho Y.W., Abdullah N., Jalaludin S. (1998). Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine. *Letters of Applied Microbiology*. 27, 183-185.

Lee Y.K., Salimen S. (1995). The coming age of probiotics. *Food Sciences and Technology.* 6, 241-245.

Tagg J.R., McGiven A.R. (1971). Assay system for bacteriocins. *Applied and Environmental Microbiology*. 21, 934.

Tanaka H., Doesburg K., Iwasaki T., Mireau I. (1999). Screening of lactic acid bacteria for bile salt hydrolase activity. *Journal of Dairy Sciences.* 82, 2530-2535.

Tannock G.W., Dashkevitz M.P., Feighner S.D. (1989). Lactobacilli and bile salt hydrolase in the murine intestinal tract. *Applied and Environmental Microbiology.* 55, 1848-1851.

Tannock G.W. (2004). A special fondness for lactobacilli. *Applied and Environmental Microbiology.*70, 3189-3194.

Xanthopoulos V., Litopoulou-Tzanetaki E., Tzanetakis N. (1997). *Lactic acid bacteria*. Caen Presses Universitaires de Canada.

Wayne, PaOuwehand A.C., Kirjavainen P.V., Shortt C., Salminen S.J. (1999). Probiotics: mechanics and established effects. *International Journal of Dairy.* 9, 43-52.

*** NCCLS (National Committee for Clinical Laboratory Standards) (1999). Performance standards for antimicrobial susceptibility testing, 9th informational supplement. Approved standard M100-S9. National Committee for Clinical Laboratory Standards