Dairy Sci. Technol. 88 (2008) 695–705
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DOI: 10.1051/dst:2008018

Available online at:
www.dairy-journal.org

Note

Characterization of probiotic properties of *Lactobacillus* strains

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Received 22 August 2007 – Accepted 25 March 2008

Abstract – The objectives of this study were to demonstrate the gastro-intestinal tolerance and the capacity to modulate the intestinal microbiota of some *Lactobacillus acidophilus, L. casei* and *L. rhamnosus* strains with high probiotic potential. *L. acidophilus* and *L. casei* mixture that is used to produce probiotic fermented milk has been evaluated for its acid resistance and bile salts tolerance and compared to other lactic acid bacteria (LAB). The commercial culture and the three strains of *L. rhamnosus* exhibited complete tolerance to acid for pH ≥ 2.5. The minimal inhibitory concentration of the bile salts mixture was 50 g·L^{-1} for all bacteria. The impact of the ingestion of the novel probiotic on the fecal microbiota was evaluated in vivo using healthy C57Bl/6 mice. Fecal samples were analyzed for the microbiota enumeration using selective plating. Fecal analysis showed an increase of total culturable LAB and a decrease in *Staphylococcus* spp. population in the LAB-treated mice indicating that these cultures could improve the intestinal health. Also, reduction in fecal *Enterobacteriaceae* was noticed following mice gavage with *L. rhamnosus* ATCC 9595 while a higher enumeration was measured for *L. rhamnosus* RW-9595M, an exopolysaccharide-overproducing mutant. These contradictory results were discussed.

probiotic / *Lactobacillus* / antimicrobial / gastrointestinal

摘要 – 益生性乳杆菌的特性。本研究证明了一些具有益生性的嗜酸乳杆菌、干酪乳杆菌和鼠李糖乳杆菌在胃肠道中的耐受性和调节肠道菌群的能力。本文评价了用于生产益生性发酵乳制品的嗜酸乳杆菌和干酪乳杆菌的混合菌株和其他乳酸菌的耐酸性和胆盐耐受性。研究证明混合菌株和 3 株鼠李糖乳杆菌均可以耐受到 pH ≥ 2.5 的酸性。所有菌株的胆酸盐最小耐受浓度是 50 g·L^{-1}。C57Bl/6 健康小鼠用于体外评价摄入益生菌后小鼠排泄物的微生物群。采用选择性平板计数法分析了粪便样品的微生物群。根据对粪便分析的结果表明, 乳酸菌灌胃后小鼠粪便中可培养乳酸菌的总数明显增加, 而葡萄球菌属的数量明显减少, 由此可以预示乳酸菌对改进小鼠肠道健康的作用。同时, 鼠李糖乳杆菌 ATCC 9595 灌胃后小鼠粪便中肠杆菌科的数量减少。然而一株产胞外多糖的鼠李糖乳杆菌 RW-9595M 变异种与其野生型菌株相比, 对粪便中微生物群的调整作用不同, 这一矛盾的结果有待进一步研究。

益生性 / 乳酸菌 / 抗菌 / 胃肠道

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Résumé – Caractérisation de propriétés probiotiques de souches de *Lactobacillus*. Les objectifs de cette étude consistaient à démontrer la survie gastro-intestinale et la capacité à moduler le microbiote intestinal de souches de *Lactobacillus acidophilus*, *L. casei* et *L. rhamnosus* présentant un fort potentiel probiotique. La résistance gastrique et aux sels biliaires d'un mélange composé de *L. acidophilus* et *L. casei*, utilisé comme ferment pour produire un lait fermenté probiotique, a été évaluée et comparée à d'autres bactéries lactiques. La culture commerciale et trois souches de *L. rhamnosus* ont montré une résistance complète à un pH ≥ 2,5. La concentration minimale inhibitrice de sels biliaires était de 50 $g{\cdot}L^{-1}$ pour toutes les bactéries. L'impact de l'ingestion du probiotique nouveau sur le microbiote fécal a été évalué in vivo sur des souris C57Bl/6 saines. Le dénombrement du microbiote intestinal a été effectué dans des échantillons de fèces par l'utilisation de géloses sélectives. Les analyses fécales ont montré une augmentation des bactéries lactiques totales cultivables et une diminution de la population de *Staphylococcus* spp. chez les souris traitées avec les bactéries lactiques indiquant que ces cultures pourraient améliorer la santé intestinale. De plus, une diminution des *Enterobacteriaceae* fécales a été remarquée suite au gavage avec *L. rhamnosus* ATCC 9595 tandis qu'un dénombrement plus élevé a été mesuré avec *L. rhamnosus* RW-9595M, un mutant qui surproduit des exopolysaccharides. Ces résultats contradictoires ont été discutés.

probiotique / *Lactobacillus* / antimicrobien / gastro-intestinal

1. INTRODUCTION

Lactic acid bacteria (LAB) play a significant role in fermented foods [12, 13] and produce antimicrobial metabolite compounds such as lactic acid, bacteriocins (e.g. acidocin, acidophilin, lacticin, nisin), and hydrogen peroxide [14]. Some strains of LAB can be considered as probiotic bacteria. Probiotics can be defined as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host [5]. Many studies have demonstrated the efficiency of probiotics to offer a proper alternative to the use of antibiotics in the treatment of enteric infection [16] or to reduce the symptoms of antibiotic-associated diarrhea [19]. A *Lactobacillus acidophilus* and *L. casei* fermented milk containing over 50 billions of live bacteria per portion of 98 g have been successfully used to prevent antibiotic-associated diarrhea and has demonstrated the potential to reduce *Clostridium difficile*-associated diarrhea at Maisonneuve-Rosemont hospital in Montréal, Québec, Canada [1].

Viability and survival of probiotic bacteria are important characteristics in order to provide health benefits. Probiotic should survive the gastro-intestinal transit to finally colonize the gut. Natural resistance to gastro-intestinal transit varies between LAB species [4]. Indeed, certain strains have the capacity to resist more easily to the extreme acidity of stomach or to the bile salts in the small intestine [6].

Another desirable characteristic of probiotics is their capacity to modulate the intestinal microbiota [19]. Although, some studies did not demonstrate that probiotic consumption can influence this complex ecosystem [22, 24] some others showed a significant difference [5, 17–19]. In 2006, Cinquin et al. [3] have studied the prebiotic effect of exopolysaccharides produced by *L. rhamnosus* RW-9595M using a three-stage chemostat containing immobilized infant fecal microflora. The purified polysaccharide was not metabolized by the infant microbiota and lacked prebiotic effect. However, there are no data reporting the capacity of this live bacterial strain to modulate the intestinal microbiota in vivo. This study will compare the ability of the exopolysaccharide-producing strain to the wild type *L. rhamnosus* ATCC 9595 and to a probiotic *L. acidophilus*/*L. casei* mixture.

The objective of this study was to evaluate some probiotic characteristics of a *L. acidophilus*/*L. casei* mixture and

L. rhamnosus ATCC 9595 and RW-9595M. The bile salts and acid tolerance of these strains and the capacity to modulate the fecal microbiota of C57Bl/6 mice were evaluated over the course of a three-week gastric inoculation trial and compared to other LAB strains.

2. MATERIALS AND METHODS

2.1. Bacterial strains

The probiotic *Lactobacillus acidophilus* CL1285 and *L. casei* mixture was provided by Bio-K+ International Inc. (Laval, QC, Canada). *Lactobacillus rhamnosus* GG ATCC 53103 was obtained from the American Type Culture Collection (Manassas, VA, USA) while *L. rhamnosus* ATCC 9595 and *L. rhamnosus* RW-9595M were provided by Agriculture and Agri-Food Canada (St-Hyacinthe, QC, Canada). Lactobacilli were propagated in Lactobacilli MRS broth (MRS, Difco Laboratories, Detroit, MI, USA) at 37 °C for 24 h. Bacterial strains were stored at –80 °C in their respective media containing 100 $g \cdot L^{-1}$ glycerol (Laboratoires MAT, Montreal, QC, Canada). Before each experiment, the bacterial content of one vial was thawed, transferred to 9 mL of MRS media and activated by two consecutive inoculations and incubation for 24 h at 37 °C. Thereafter, bacteria were washed twice in sterile saline (8.5 $g \cdot L^{-1}$) after centrifugation at 4 °C for 10 min at 6000× *g*.

2.2. Acid tolerance of LAB

Simulated gastric fluid (SGF) was formulated according to *United States Pharmacopeia* (USP) [11]. Briefly, SGF was composed by 3.2 $g \cdot L^{-1}$ of pepsin (Sigma, Oakville, ON, Canada), 2.0 $g \cdot L^{-1}$ NaCl and pH was finally adjusted to 1.5, 2.0, 2.5 or 3.0 by addition of HCl (5 $mol \cdot L^{-1}$). A volume of 1 mL of an overnight MRS broth culture of LAB was added to 19 mL of SGF for 30 min at 37 °C under mild agitation (200 rpm) in a G24 Environmental Incubator Shaker (New Brunswick Scientific Co. Inc., NJ, USA). After 30 min in gastric solution, 1 mL was collected and mixed in 9 mL of sterile phosphate buffer saline (PBS; pH 7.4). A similar process was carried out for bacteria without the acidic treatment in order to determine the initial concentration of LAB.

2.3. Viable cell determination

Appropriate dilutions from these samples were done in sterile peptone water (1 $g \cdot L^{-1}$) and plated (pour-plate method) on MRS agar. Plates were incubated under aerobic conditions at 37 °C for 48 h. The average number of colony-forming units (cfu) from triplicate analysis was determined using a Darkfield Quebec Colony Counter.

2.4. Bile salt tolerance of LAB

The bile salt tolerance of probiotic LAB was determined using a method described by Casey et al. [2]. Briefly, MRS agar containing a commercial preparation of bile salts normally used to inhibit growth of Gram positive bacteria in broth, bile salts mixture (Sigma B-3426, Oakville, ON, Canada) was added in concentrations varying between 0 and 100 $g \cdot L^{-1}$. Bile salts containing-MRS agar was then autoclaved for 15 min at 121 °C, cooled and plated. Overnight MRS broth cultures (100 µL of bacteria in the stationary phase of growth) were inoculated on surface of bile salts-containing MRS agar and incubated at 37 °C for 72 h under anaerobic conditions. Presence of bacterial lawn indicated a good growth and thus good resistance of bacteria to bile salts while presence of small and isolated colonies indicated a poor resistance to bile salts. Absence of colonies

indicated that the LAB did not tolerate the bile salt concentration assayed. Minimal inhibitory concentration represented the lowest concentration of the bile salts assayed totally inhibiting the growth of colonies as judged from visual examination.

2.5. Animals

Six- to eight-week-old female C57Bl/6 mice were housed in plastic cages and kept under pathogen-free conditions with free access to commercial chow and water. This work was approved and supervised by the INRS-IAF Animal Care Committee.

2.6. Intragastric administration of LAB to mice

Four healthy mice received a daily dose of about 10^9 viable bacteria (CL1285 mixture, *L. rhamnosus* ATCC 9595 or *L. rhamnosus* RW-9595M) in 100 μL of PBS (pH 7.2) by intragastric route using a stainless steel feeding needle and a 1-mL syringe. Mice were weighed at day 1, 9, 18, and then 9 days after the end of the treatment (day 27-post gavage) and any signs of physiological or psychological perturbation were noticed along the experiment. Stool samples were collected before the administration of PBS or probiotics and at day 1, 9 and 18 after the beginning of the administration procedures. Final analysis was done 9 days after the end of the treatment (day 27-post gavage). Two independent repetitions were done for a total of eight mice in each experimental group.

2.7. Quantification of stool microorganisms

Fresh stool samples were weighed, diluted in 1 mL of sterile saline, homogenized with a pestle, 10-fold serially diluted in peptone water and finally 100 μL were inoculated on the following media: MRS agar for detection of total lactic acid bacteria (LAB), Rogosa SL agar for detection of *Lactobacillus* spp., Reinforced Clostridium Medium (RCM) for quantification of total anaerobic mesophilic bacteria, Baird-Parker agar (BPA) for detection of *Staphylococcus* spp. and MacConkey agar for enumeration of *Enterobacteriaceae*. MRS, Rogosa and RCM plates were incubated in anaerobic jars at 37 °C for 72 h while BPA and MacConkey plates were incubated under aerobic conditions at 37 °C for 48 h. The lower limit of detection was 10^2 microorganisms per mg feces.

2.8. Statistical analyses

Acid resistance experiments were carried out in triplicate. For each replication, three samples were analyzed. Student-*t* test was done using statistics SPSS program (version 10.1) to determine if there is a significant difference of viability between LAB population before and after acid treatment. Finally, analysis of variance (ANOVA) was done to verify if there is a significant difference of microbial population in feces of mice fed with various probiotics. Data were analyzed at a 5% level of significance.

3. RESULTS

3.1. Survival of LAB to simulated gastric fluid and growth in presence of bile salts

The growth of LAB in MRS in presence of an increasing concentration of bile (0–100 $g \cdot L^{-1}$) salts was evaluated in order to verify the tolerance of the probiotic CL1285 to bile. The minimal inhibitory concentration of the bile salts mixture was 50 $g \cdot L^{-1}$ for both bacteria

Table I. Survival of lactic acid bacteria strains after an incubation of 30 min at 37 °C in simulated gastric fluid (pH 1.5 to 3.0).

Microorganisms	Time (min)	pH	Log cfu survivor
L. acidophilus/*L. casei* (CL1285)	0	–	$9.57 \pm 0.09^{B*}$
	30	1.5	< 1
	30	2.0	5.90 ± 0.52^{A}
	30	2.5	9.55 ± 0.04^{B}
	30	3.0	9.63 ± 0.04^{B}
L. rhamnosus GG ATCC 53103	0	–	9.10 ± 0.13^{B}
	30	1.5	< 1
	30	2.0	5.33 ± 0.62^{A}
	30	2.5	9.08 ± 0.14^{B}
	30	3.0	9.01 ± 0.13^{B}
L. rhamnosus ATCC 9595	0	–	9.57 ± 0.09^{B}
	30	1.5	< 1
	30	2.0	5.09 ± 0.77^{A}
	30	2.5	9.51 ± 0.10^{B}
	30	3.0	9.51 ± 0.09^{B}
L. rhamnosus RW-9595M	0	–	9.69 ± 0.09^{B}
	30	1.5	< 1
	30	2.0	4.32 ± 1.27^{A}
	30	2.5	9.84 ± 0.11^{B}
	30	3.0	9.81 ± 0.18^{B}

*: Within each bacterial group, means with common superscripts do not differ ($P \leq 0.05$).
Mean log cfu and standard deviation from three independent repetitions are presented.

(data not shown). Moreover, results presented in Table I show that all bacteria tested can survive at an acidic environment during 30 min. No significant difference ($P > 0.05$) was observed between initial microbial population at 0 and 30 min for pH $\geq$ 2.5 while significant reduction was observed at pH 2 for all bacteria. Reduction in bacterial population of 5.37, 4.48, 3.77 and 3.67 log cfu were observed for *L. rhamnosus* RW-9595M, *L. rhamnosus* ATCC 9595, *L. rhamnosus* GG and the *L. acidophilus*/*L. casei* mixture, respectively. No bacterial viability was detected after 30 min at pH 1.5.

3.2. Modulation of the fecal microbiota

The composition of microbial populations in C57Bl/6 mice fecal samples during the trial is shown in Figures 1–4. The LAB counts in the mice feces were significantly higher after 18 d of CL1285 ingestion than after PBS ingestion ($P \leq 0.05$). However, after administration ended, the level of LAB was similar to its initial count. Ingestion of *L. rhamnosus* ATCC 9595 led to a significant decrease of LAB count ($P \leq 0.05$) after 9 d of LAB administration while an increase was observed

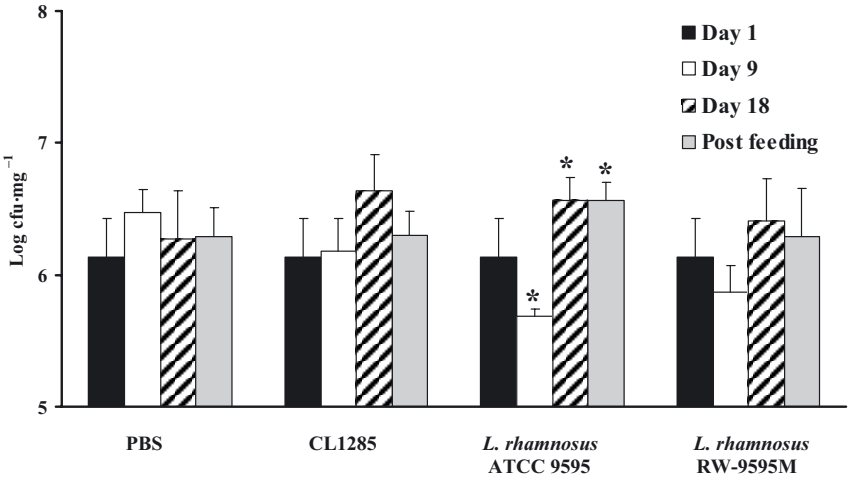

Figure 1. Lactic acid bacteria population in feces of C57Bl/6 mice during gastric inoculation with probiotic bacteria. Error bars represent the standard deviation of the mean log cfu·mg^{-1} feces obtained from eight mice in two independent experiments. *: Variations are considered significant ($P \leq 0.05$) when bacterial concentration from a given day was different from Day 1 of the same group and from the bacterial concentration of the PBS group of the same day.

after 18 d. The high level of LAB population was also observed after gavage ended (Fig. 1). The same observations were noticed in mice fed with *L. rhamnosus* RW-9595M but LAB count variations were not statistically significant as compared to the PBS group. *Lactobacillus* spp. population were not affected significantly ($P > 0.05$) by the presence of all probiotics evaluated (data not shown). As observed for lactobacilli, fecal *Enterobacteriaceae* counts were not affected quantitatively by the presence of CL1285 or *L. rhamnosus* RW-9595M (Fig. 2). However, after 18-d of gavage with *L. rhamnosus* ATCC 9595, a reduction of 0.43 log cfu·mg^{-1} was observed. This reduction was only temporary as seen by the increase in *Enterobacteriaceae* population observed after the oral administration ended (Fig. 2). The Figure 3 shows that the consumption of the CL1285 reduced the *Staphylococcus* spp. population after 18 d and over of gavage while the variation in *Staphylococcus* spp. population was seen after nine days of gastric inoculation with both *L. rhamnosus* but was only temporary. Finally, Figure 4 shows the total anaerobe counts in feces of each group of mice. An increase of these populations was observed for both *L. rhamnosus* strains treated groups ($P \leq 0.05$). This increase was observed until the end of the treatment for the ATCC 9595 strain but not for the RW-9595M strain after the post-gavage.

4. DISCUSSION

LAB have been used since a long period time in food fermentation intended for human consumption. A persistent problem of probiotics is their ability to resist, survive or colonize the intestine at least temporarily. LAB must survive the acidic environment of the stomach in order to reach the gut and modulate the microbiota. Results obtained in this study demonstrated

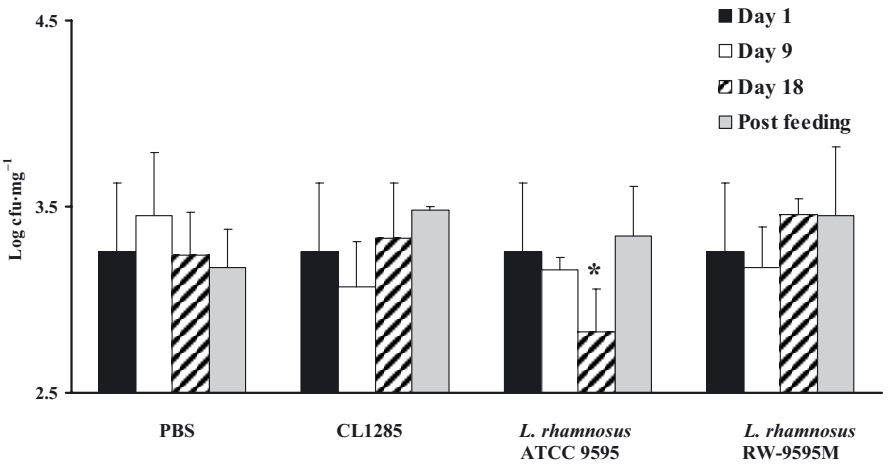

Figure 2. *Enterobacteriaceae* population in feces of C57Bl/6 mice during gastric inoculation with probiotic bacteria. Error bars represent the standard deviation of the mean log cfu·mg^{-1} feces obtained from eight mice in two independent experiments. *: Variations are considered significant ($P \leq 0.05$) when bacterial concentration from a given day was different from Day 1 of the same group and from the bacterial concentration of the PBS group of the same day.

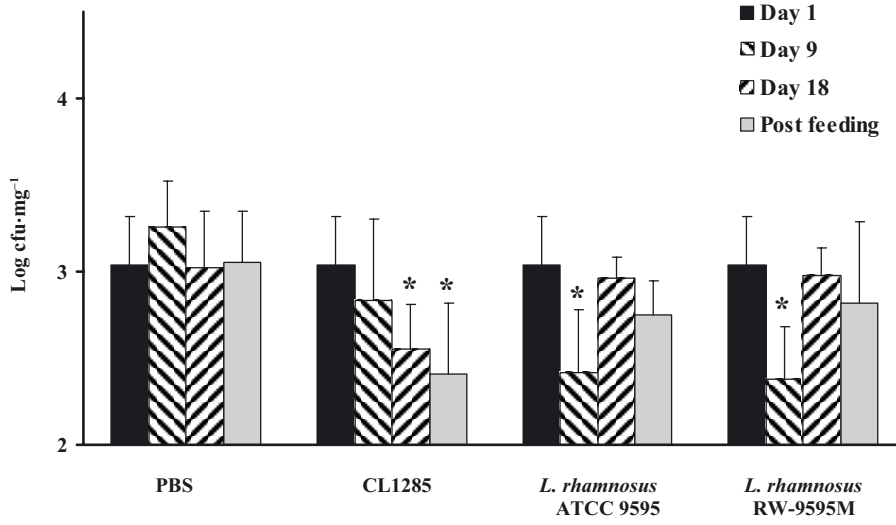

Figure 3. *Staphylococcus* spp. population in feces of C57Bl/6 mice during gastric inoculation with probiotic bacteria. Error bars represent the standard deviation of the mean log cfu·mg^{-1} feces obtained from eight mice in two independent experiments. *: Variations are considered significant ($P \leq 0.05$) when bacterial concentration from a given day was different from Day 1 of the same group and from the bacterial concentration of the PBS group of the same day.

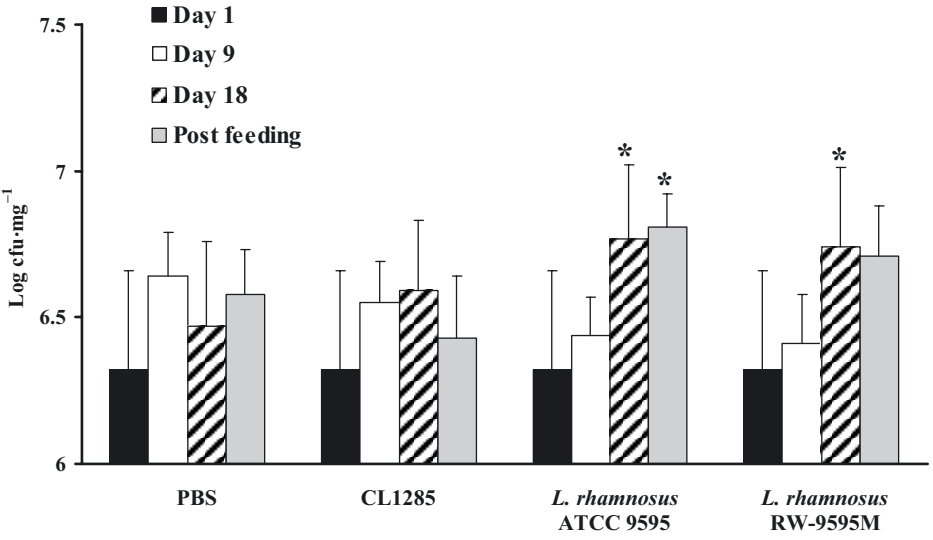

Figure 4. Content of the total mesophilic anaerobes population in feces of C57Bl/6 mice during gastric inoculation with probiotic bacteria. Error bars represent the standard deviation of the mean log cfu·mg^{-1} feces obtained from eight mice in two independent experiments. *: Variations are considered significant ($P \leq 0.05$) when bacterial concentration from a given day was different from Day 1 of the same group and from the bacterial concentration of the PBS group of the same day.

that the CL1285 probiotic preparation have a complete resistance under simulated gastric fluid at pH ≥ 2.5, which is the pH of the stomach following food consumption [6]. Moreover, CL1285 and *L. rhamnosus* GG seem to have the highest resistance at pH 2 as compared with other *L. rhamnosus* evaluated in this study. A 3.7 log cfu reduction was observed as compared to 5 log cfu reduction for other *L. rhamnosus*. However, the differences were not significant. Bile salt tolerance is considered one of the most important attributes required by LAB to survive in the duodenum and the upper small intestine [20]. Our study showed that CL1285 and *L. rhamnosus* GG survived a bile salt stress but also grew on MRS agar containing 4% of a standardized bile salts mixture. However, at this point, it is not possible to confirm that the strains present in the CL1285 mixture have the same acidity and bile salt tolerance. Succi et al. [21] observed a 7 log cfu·mL^{-1} reduction when *L. rhamnosus* GG was placed in acidified MRS (pH 2) after an incubation of 2 h at 37 °C while a reduction of approximately 2.5 log cfu·mL^{-1} was measured at pH 3. Subsequently, the bacterial culture was transferred in MRS at pH 7 but containing 20 g·L^{-1} of bile salts (Oxoid LP0055; Basingstoke, UK) for 5 h at 37 °C. *L. rhamnosus* GG showed no loss of viability at the end of the treatment which is in agreement with results obtained in this study.

This study also demonstrated that ingestion of novel probiotics is well tolerated by C57Bl/6 mice over the course of a three week-administration trial and can alter quantitatively the balance of colonic bacterial populations. This effect is strain dependant. All the probiotic tested have the potential to increase, at least transiently, the total culturable LAB content. Although

the mice were fed routinely with probiotic *Lactobacillus* strains, the increase in total LAB was not correlated with an elevation of the *Lactobacillus* population. It could be hypothesized that a reorganization of the intestinal microbiota was induced and the probiotic species replaced or stimulated the growth of the indigenous *Lactobacillus* strains leading to a variation of the bacterial species but the total culturable *Lactobacillus* spp. enumeration on Rogosa agar was not modified. Manninen et al. [15] observed that indigenous *L. acidophilus* population in the small intestine of dogs was stimulated following ingestion of other species of the *Lactobacillus* genera. The same hypothesis could explain the increase in total anaerobes obtained following ingestion of both *L. rhamnosus* strains. However, there was no variation in total anaerobes following the ingestion of the CL1285. MRS agar and Rogosa SL are media usually used in numerous studies for the enumeration of total LAB or *Lactobacillus* spp. in human or animal feces or gut [7, 10]. However, these media also permit the growth of non-LAB such as many species of *Bifidobacterium* spp. or *Pediococcus* spp. for the Rogosa SL agar [8]. This could explain the elevated concentration of *Lactobacillus* spp. in regard of the total anaerobe counts. We also studied bacterial populations that are less abundant in intestinal microbiota such as total *Enterobacteriacea* and *Staphylococcus*. These microbial populations are often considered as deleterious microorganisms [9]. For *Enterobacteriacea* population, only ingestion of *L. rhamnosus* ATCC 9595 by mice decreased the counts of culturable *Enterobacteriaceae*, while the other probiotics did not modulate quantitatively this population. This reduction correlated with an increase of the total LAB and anaerobes. However, the level of *Enterobacteriaceae* counts was similar to its initial level after probiotic intragastric administration ended. This study has shown that *L. rhamnosus* RW-9595M did not have the same effect as the *L. rhamnosus* ATCC 9595 wild-type strain on the *Enterobacteriacea* population. Further experiments will be needed to have a better comprehension of this phenomenon. A reduction of *Staphylococcus* spp. was noticed following the ingestion of CL1285. It could be hypothesized that CL1285 culture inhibits the growth of *S. aureus* as shown by the in vitro experiments. The reorganization of the microbial population could explain the temporary decrease in *Staphylococcus* sp. observed for both *L. rhamnosus* evaluated. It could thus be suggested that the composition of intestinal microbiota could be altered specifically following ingestion of probiotics. Overall, in order to ascertain these hypothesis properly, culture-independent techniques such as the integrated use of denaturated gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH) and real time-PCR should be used to monitor qualitatively and quantitatively the variations in fecal microbial populations during probiotic administration. These techniques also have the advantage to be more specific and sensitive than plate counts on selective media [17, 23].

5. CONCLUSION

This study has demonstrated that the novel probiotic CL1285 and other probiotic could survive the stressful gastrointestinal transit and reach the gut, and their resistance is significantly higher than other bacteria tested. This study also showed that mice supplementation with probiotic bacteria had an effect on the intestinal microbiota. The population of total LAB in the feces increased while *Staphylococcus* sp. decreased following the gavage of mice with all probiotics evaluated. Moreover, this study showed that an exopolysaccharide-producing strain of

L. rhamnosus modulate differently the fecal microbiota as compared to its wild-type strain. Further studies using molecular tools should give more information.

Acknowledgements: This research was supported by Quebec Research Council in Fishing and Agri-Food (CORPAQ) of the Quebec ministry of Agriculture, Fishery and Food (MAPAQ) and by Bio-K Plus International Inc. through their "Volet Compétitivité des Entreprises" program. The authors are grateful to Bio-K Plus International Inc. for the CL1285 probiotic preparation and to Agriculture Canada for the *L. rhamnosus* strains.

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