Zebrafish gut colonization by mCherry-labelled lactic acid bacteria

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18 **Abstract**

19 A critical feature of probiotic microorganisms is their ability to colonize the intestine of the host.
20 Although the microbial potential to adhere to the human gut lumen has been investigated in *in vitro*
21 models, there is still much to discover about their *in vivo* behaviour. Zebrafish is a vertebrate model
22 that is being widely used to investigate various biological processes shared with humans. In this
23 work we report on the use of the zebrafish model to investigate the *in vivo* colonization ability of
24 previously characterized probiotic lactic acid bacteria. *Lactobacillus plantarum* Lp90, *L. plantarum*
25 B2 and *Lactobacillus fermentum* PBCC11.5 were fluorescently tagged by transfer of the pRCR12
26 plasmid, which encodes the mCherry protein and which was constructed in this work. The
27 recombinant bacteria were used to infect germ-free zebrafish larvae. After removal of bacteria, the
28 colonization ability of the strains was monitored until 3 days post-infection by using a fluorescence
29 stereomicroscope. The results indicated differential adhesion capabilities among the strains.
30 Interestingly, a displacement of bacteria from the medium to the posterior intestinal tract was
31 observed as a function of time that suggested a transient colonization by probiotics. Based on
32 fluorescence observation *L. plantarum* strains exhibited a more robust adhesion capability. In
33 conclusion, the use of pRCR12 plasmid for labelling *Lactobacillus* strains provides a powerful and
34 very efficient tool to monitor the *in vivo* colonization in zebrafish larvae and to investigate the
35 adhesion ability of probiotic microorganisms.

36

37 **Keywords:** zebrafish, probiotic, *in vivo*, *Lactobacillus plantarum*, *Lactobacillus fermentum*

38 **1. Introduction**

39 Lactic Acid Bacteria (LAB) are key microorganisms in the food industry, because they have been
40 used for centuries for the production of fermented beverages, dairy, meat and vegetables. Alongside
41 such traditional applications, in recent years LAB are gaining a pivotal role in the health industry
42 for their probiotic features (Saxelin 2008; Douillard and de Vos 2014). Due to this trend a number
43 of *Lactobacillus* species have been selected and commercialized as live microorganisms for food
44 supplementation (Foligné et al. 2013). The health-promoting effects of probiotics are related to their
45 ability to colonize the host gut and they contribute to nutritional physiology, modulation of gut
46 mucosal immunity and prevention, or treatment, of intestinal infections (Marco et al. 2006; Turpin
47 et al. 2010; Bron et al. 2012). Therefore, a crucial prerequisite to select potential probiotic strains is
48 that their adhesion to the intestinal epithelium is high enough to confer health benefits (de Vrese
49 and Schrezenmeir 2008). Nowadays, advances in sequencing have provided an increased
50 availability of known LAB genomes allowing the more rapid screening of probiotic candidates
51 based on omic approaches (Turpin et al. 2011; Sánchez et al. 2013). Thus, a number of molecular
52 markers have been investigated over the years that are involved in tolerance to typical intestinal
53 stresses such as high bile salts concentrations, binding to host surface, competition phenomena or
54 induction of proinflammatory modulators (Lebeer et al. 2008). In addition, several *in vitro* models
55 have been proposed to predict the probiotic aptitude of microbial strains (Mainville et al. 2005;
56 Fernández de Palencia et al. 2008; Bove et al. 2013). In particular, Caco-2 or HT29 human colon
57 carcinoma cell lines are simple and well established models that simulate the direct host-microbe
58 cellular contact. Moreover, mucus-secreting cultures or immobilized mucus models are employed to
59 investigate microbe-mucus and -muco-oligosaccharide interactions (van Tassell and Miller 2011).
60 However, although the microbial potential to colonize the human gut lumen has been thoroughly

61 investigated in *in vitro* models, there is still much to discover about the *in vivo* behaviour of
62 probiotics.

63 In recent years, zebrafish (*Danio rerio*) has emerged as a model to study vertebrate development,
64 immunity and disease because of their small size, high fecundity, rapid development, optical
65 transparency of the embryos, amenability to genetic screens, and structural similarities to mammals
66 (Meeker and Trede 2008; Sullivan and Kim 2008). This model has been used to study the host
67 immune response under a number of microbial infections (van der Sar et al. 2004; Rojo et al. 2007)
68 as well as the interactions between the host and its natural gut microbiota (Milligan-Myhre et al.
69 2011), as well as probiotics (Gioacchini et al. 2010; Gioacchini et al. 2011a; Gioacchini et al.
70 2011b; Rendueles et al. 2012; Carnevali et al. 2013; Rieu et al. 2014).

71 The interpretation of host-probiotics interactions in animals is complex due to the diversity of
72 microorganisms residing in the host gut. These difficulties may be overcome using laborious
73 gnotobiotic models where the microbiota is either known or absent. The use of zebrafish provides a
74 number of advantages that simplify the generation of gnotobiotic organisms as compared to
75 mammalian models. For example, as zebrafish eggs are fertilized externally and the development of
76 embryos occurs within their protective chorions, the axenic conditions can be easily conserved.

77 In this work we constructed the pRCR12 plasmid containing a synthetic *mrfp* gene (encoding the
78 monomeric red fluorescence protein - 'mCherry'), whose codon usage was optimized for LAB
79 (García-Cayuela et al. 2012). This plasmid was used to generate recombinant strains of three
80 previously characterized probiotic LAB strains which, in turn, were used to monitor the intestinal
81 colonization of gnotobiotic zebrafish larvae.

82

83 **2. Materials and Methods**

84 **2.1 Bacterial strains and growth conditions**

85 The microbial **probiotic** strains used were *Lactobacillus plantarum* Lp90, whose genome has
86 recently been sequenced (Accession number JIBX000000000), and the riboflavin over-producing
87 strains *L. plantarum* B2 and *Lactobacillus fermentum* PBCC11.5 (11.5), which have been deposited
88 in the Spanish Culture Type Collection (CECT, Valencia, Spain) with the code numbers CECT
89 8328 and CECT 8448, respectively (Arena et al. 2014; Russo et al. 2014). These strains were
90 routinely grown in MRS broth (Oxoid, Basingstoke, UK) at 37 °C. The corresponding strains
91 carrying the pRCR12 plasmid were grown on MRS broth containing chloramphenicol at 10 µg mL⁻¹.
92 ¹. In addition, *S. pneumoniae* JNR7/87 (Bricker and Camilli 1999) was used as source of
93 **chromosomal DNA** and was grown in AGCH medium (Lacks 1968) supplemented with 0.25%
94 yeast extract and 0.8% sucrose at 37 °C.

95 **2.2 Chromosomal DNA preparations**

96 Cultures of *S. pneumoniae* JNR7/87 were grown to an absorbance of 0.7 at 650 nm. Samples of 1.5 ml of the
97 cultures were sedimented and resuspended in 100 µl of a solution containing 730 mM sucrose, 150 mM
98 sodium citrate, 2.3 mM sodium deoxycholate, SDS at 0.01% and 8 µg de RNase I. Cells were lysed by
99 incubation at 37 °C for 15 min. Then, crude extracts were passed through a needle (25G 5/8 0,5X16 mm) to
100 reduce their viscosity. Then, samples were deproteinated by two extractions with 100 µl of a mixture of
101 phenol : chloroform (1:1 v/v) containing 4% isoamyl alcohol, DNA was precipitated in the presence of 0.3 M
102 sodium acetate pH 7.0 with absolute ethanol and finally resuspended in 100 µl of 10:1 TE buffer (10 mM
103 Tris pH 8.0 and 1 mM EDTA) and keep at -70 °C. 1 µl of the extracts was used for PCR reactions.

104 **2.3 Construction of the pRCR12 plasmid**

105 The pRCR12 plasmid (Fig. 1) constructed in this work is based on the pRCR plasmid, whose
106 detailed construction will be described elsewhere (Mohedano et al. paper in preparation). The

107 pRCR plasmid carries the replication origin from the lactococcal plasmid pSH71, the *cat* gene
108 responsible for chloramphenicol resistance and a synthetic *mrfp* gen codon optimized for LAB
109 (García-Cayuela et al. 2012), which encodes the monomeric mCherry fluorescent protein. pRCR12
110 (Fig. 1) was constructed by insertion of the strong P_x pneumococcal promoter of the *malXCD*
111 operon (Nieto et al. 1998) into the pRCR plasmid upstream of *mrfp* (GenBank ID: KP182347)
112 generating the transcriptional fusion P_x-*mrfp* in pRCR12. To this end, a 0.5 kb amplicon (GenBank
113 ID: KP182346) was generated by use of chromosomal DNA of *Streptococcus pneumoniae* JNR7/87
114 (Bricker and Camilli 1999) as template and ForP_x (5'-
115 GGAAGATCTCTTTTCAAGCATAATTGCAAGCG-3') and RevP_x (5'-
116 CTAGTCTAGACCTCCTAAAGAATAGCAAGTTTTATTG-3') primers. After digestion with
117 *Bgl*II and *Xba*I, the amplicon was introduced into the multicloning site of pRCR, previously
118 digested with the same restriction enzymes. The ligation mixture was used to transform competent
119 *Escherichia coli* DH5α cells and transformants, carrying the pRCR12 plasmid, were selected by
120 chloramphenicol resistance and detected on Luria Broth agar (Oxoid) by the red colour of the
121 colonies. The presence of the transcriptional fusion in pRCR12 was confirmed by DNA sequencing
122 with ForP_x and RevmRFP primers.

123 **2.4 Transfer of pRCR12 to *Lactobacillus* strains**

124 Competent *L. plantarum* Lp90, *L. plantarum* B2 and *L. fermentum* PBCC11.5 bacteria were
125 prepared as follows. The cultures were grown until they reached an OD₆₀₀ = 0.6, cooled on ice for
126 15 min with shaking every 2-3 min and centrifuged at 3,000 x g for 10 min at 10 °C. The recovered
127 pellet was washed with 1 volume of cold 1 mM MgCl₂ and centrifuged as above. The pellet was
128 washed with 1 volume of cold 30% polyethylene glycol (PEG) 1500 and after centrifugation was
129 resuspended in 1/100 volume of 30% PEG 1500.

130 For the electroporation procedure, 50 μL of the cell suspension and 5 μL of pRCR12 plasmid DNA
131 ($100 \text{ ng } \mu\text{L}^{-1}$) were mixed in a previously cooled Gene Pulser 0.1 cm cuvette (Bio-Rad).
132 The electroporation was performed using a Gene Pulser Xcell with Shock Pod Cuvette Chamber
133 (Bio-Rad, Hercules, CA) using the following parameters: voltage 1,500 V, resistance 400 Ω ;
134 capacitance 25 μF . After the electrical pulse 500 μL of MRS containing 1 mM MgCl_2 and 0.3 M
135 sucrose were immediately added and cells were incubated at 37 $^{\circ}\text{C}$ for 2 h. Transformants
136 containing plasmid pRCR12, were selected on MRS agar containing chloramphenicol at $10 \mu\text{g mL}^{-1}$
137 and detected by the red colour of the colonies.

138 **2.5 Determination of plasmid copy number**

139 Cultures of LAB strains carrying the pRCR12 plasmid were grown to an $\text{OD}_{600} = 1$. In order to
140 obtain a genomic (chromosomal and plasmidic) DNA preparation, 1 mL of each culture was
141 harvested by centrifugation at $14,000 \times g$ during 10 min at 4 $^{\circ}\text{C}$ and washed twice with phosphate
142 buffered saline (PBS) pH 8.0 (10 mM Na_2HPO_4 , 1 mM KH_2PO_4 , 140 mM NaCl and 3 mM KCl).
143 The pellets were resuspended in 100 μL lysis buffer (20% sucrose, 10 mM EDTA, RNase at 8 mg
144 mL^{-1} , mutanolysin at $240 \text{ U } \mu\text{L}^{-1}$ and lysozyme at 30 mg mL^{-1}). After 15 min incubation at 37 $^{\circ}\text{C}$,
145 SDS was added to a final concentration of 1%. Then, crude extracts were passed through a needle
146 (25G 5/8 0.5 x 16 mm) to reduce their viscosity. Then, samples were deproteinated by two
147 extractions with 100 μL of a mixture of phenol:chloroform (1:1 v/v) containing 4% isoamyl
148 alcohol. Afterwards, DNA was precipitated in the presence of 0.3 M sodium acetate pH 7.0 with 2.5
149 volumes of absolute ethanol and finally resuspended in 100 μL of 10 mM Tris-HCl pH 8.0. The
150 genomic DNA preparations were fractioned in a 1.2% agarose gel, the gel was stained with
151 ethidium bromide at $0.5 \mu\text{g mL}^{-1}$ for 1 h and the intensity of the DNA bands was quantified with a
152 Gel Doc 2000 instrument and the Quantity One 4.6.5 software (Bio-Rad).

153 The average plasmid copy number (from three determinations) was calculated using the following
154 equation developed by Projan et al. (1983):

155
$$N = \frac{(1.36Dp1) \times M_c}{D_c \times M_p}$$

156 where DP1 is the intensity value determined for the covalently closed plasmid forms. M_c is the
157 molecular weight of the *L. plantarum* WCFS1 chromosome (3,308,274 bp) or the *L. fermentum*
158 CECT5716 chromosome (2,100,449 bp) and D_c is the intensity value for the chromosomal DNA.
159 M_p is the molecular weight of the plasmid pRCR12 (4,600 bp). The coefficient 1.36 is introduced to
160 correct for the differences in fluorescence due to the efficiency of ethidium bromide to intercalate
161 with linear and supercoiled DNA.

162 **2.6 Simultaneous determination of cell growth and fluorescence**

163 The recombinant cultures were diluted in MRS containing $10 \mu\text{g mL}^{-1}$ of chloramphenicol to OD_{600}
164 $= 0.05$, and $200 \mu\text{L}$ of each culture were placed in triplicate in Costar 96 - Well EIA/RIA Plate
165 stripwells (ImmunoChemistry Technologies, Bloomington, MN). The levels of fluorescence of the
166 mCherry protein and bacterial growth were tested simultaneously with the Varioskan Flash system
167 (Thermo Fisher Scientific, Waltham, MA), which provides quantitative data of cell density by
168 measuring the OD at 600 nm and mCherry expression at an excitation wavelength of 587 nm and an
169 emission wavelength of 612 nm.

170 **2.7 Zebrafish husbandry**

171 Zebrafish embryos were obtained from wild type adult zebrafish (*Danio rerio*, Hamilton 1822) bred
172 in the AZTI Zebrafish Facility (REGA Number ES489010006105; Derio, Spain) following standard
173 conditions. Fish were maintained at 27°C in 60 L tanks, with aerated freshwater. Each tank
174 contained an external filtration system (biological, chemical and physical filtration), supplemented
175 by an ultraviolet lamp. Zebrafish were maintained according to standard protocols (Nüsslein-
176 Volhard and Dahm 2002). Fish were fed with a pellet formulated diet (Gemma Micro 300,

177 Skretting) to achieve a total daily feed input of 5% of body weight per day and were reared on a 12-
178 h light/12-h dark cycle. All experimental procedures were approved by the Regional Animal-
179 Welfare Body.

180 **2.8 Disinfection of zebrafish embryos**

181 Embryos were collected directly from the breeding tanks immediately after fertilization. From this
182 first step the entire procedure was carried out in a laminar flow cabinet to maintain sterile
183 conditions; sterile solutions and materials were also used. The fertilized embryos were washed with
184 a sterilized EWB solution [Embryo water (EW): CaCl_2 294 mg mL^{-1} , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at 123.3 mg
185 mL^{-1} , NaHCO_3 at 63 mg mL^{-1} , KCl at 5.5 mg mL^{-1} and supplemented with methylene blue 0.01 %
186 (w/v)] to remove the faecal matters. Embryos were carefully collected with a sterile disposable
187 plastic pipette and moved to a 15 mL sterilized conical tube and washed ten times in EWB. At this
188 point, embryos with visible debris attached to the chorion were rejected (Pham et al. 2008). After
189 the first washing step, embryos were washed ten times in antibiotic solution (AB solution). The AB
190 solution contained three antibiotics at the following final concentrations; kanamycin at 15 $\mu\text{g mL}^{-1}$,
191 ampicillin at 300 $\mu\text{g mL}^{-1}$ and amphotericin B at 1.25 $\mu\text{g mL}^{-1}$. This washing step, and the
192 composition of the washing solution, is a crucial factor and we modified the protocol previously
193 described by Pham et al. (2008) by increasing the final concentrations of the antibiotics. After that,
194 the AB solution was removed and embryos were gently immersed in 0.02 % (w/v)
195 polyvinylpyrrolidone (PVP) for exactly 2 min. The PVP was then removed by washing the embryos
196 ten times in EWB. The treatment timing and final concentration were strictly controlled, because
197 PVP is extremely toxic to aquatic life. After PVP treatment the embryos were incubated in a 0.003
198 % (v/v) bleach solution during 1 hour and finally washed ten times in sterile EWB solution. Dead
199 embryos were removed during this process in order to minimize the growth of microorganisms.
200 Subsequently, the embryos were incubated overnight in AB solution containing 75 μM 1-phenyl-2-

thiourea (PTU) to inhibit pigment development (Karlsson et al. 2001). Thereafter, all media contained PTU to maintain the inhibition. The following day, the AB solution was removed by washing the embryos ten times in sterile EWB solution. Then, 50 embryos were collected and transferred to a Petri plate (5.5 cm diameter $\times$ 1.0 cm) to be immersed in 5 mL EWB solution and treated with two UV-light pulses of 1.6 kV (Pulsed UV System XeMatica 1:2L-SA, SteriBeam Systems, GmbH) to inactivate the microbial burden present in the sample.

2.9 Axenity test

After the Light Pulse treatment and at 96 hours post fertilization the axenity was tested. Water from the obtained germ-free zebrafish embryos was tested to assess its sterility by the routine culturing of 1 mL of water on a non-selective microbiological growth medium, Plate Count Agar (Oxoid) and brain/heart infusion broth (Oxoid) at 30 °C during 48-72 h to monitor total and viable bacterial growth. Samples were also cultured on 10 mL of Sabouraud Dextrose Broth (Sab-Dex) and incubated at 25 °C during 3-5 days, to detect the presence of yeasts, moulds and acidophilic microorganisms. Additionally, PCR amplification using primers targeting 16S ribosomal RNA gene were carried out to determine the presence of any bacteria inside the larvae.

2.10 *Lactobacillus* culture conditions and challenge test

The recombinant LAB carrying pRCR12 were grown in MRS broth supplemented with chloramphenicol at 10 $\mu\text{g mL}^{-1}$ and 0.05% cysteine. Overnight cultures were washed three times in PBS (pH 7.0) at room temperature and then diluted to 10^7 CFU mL^{-1} . Ten millilitres of each of the dilutions were poured into Petri dishes (5.5 cm diameter $\times$ 1.0 cm). Using a Pasteur pipette, between 10 and 15 gnotobiotic zebrafish larvae of 4 dpf were placed into each of the Petri dishes and incubated at 27 °C with shaking (90 rpm). Fifteen gnotobiotic larvae were placed in PBS as control. After 18 h, the medium was removed and the larvae were washed three times with PBS. After 6, 24, 48 and 72 h, individual larvae were examined visually using a Leica MZFL III

225 stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) with a zoom magnification
226 range of 8 x to 100 x. The microscope was equipped with a visible light and an ultraviolet light (Hg
227 100 W) source. mCherry fluorescence was detected by exposure of the larvae to ultraviolet light in
228 the excitation range of 540-580 nm. Images were captured using a Leica DFC 360FX camera and
229 processed with the LAS-AF (Leica Microsystems GmbH) and ImageJ. Intestinal enterocytes were
230 visualized after larvae anaesthetization using tricaine (MS-222) (Sigma Aldrich, St Louis, MO) at
231 200 mg mL⁻¹ and identified with an Olympus Fluoview FV500 confocal microscope. The images
232 were acquired using a Plan Apo 20x/0.70 objective with an excitation wavelength of 587nm. The
233 images were analysed using Olympus FLuoView 5.0.67 and ImageJ 1.48 software.

234 **2.1.1 Enumeration of probiotic LAB in infected zebrafish larvae**

235 Zebrafish larvae were euthanized with tricaine (MS-222) (Sigma Aldrich) at 200 mg mL⁻¹. Then,
236 they were washed in 3 different baths of sterile PBS-0.1 % (v/v) and Tween 20 to remove the
237 bacteria loosely attached to the skin. In each experimental condition 15 larvae were manually and
238 individually homogenized with a Pellet Pestle Cordless Motor (Kimble Chase, Vineland, NJ) in 500
239 µL of PBS. Finally, serial dilutions of the recovered suspension were spotted on to plates. Colony
240 forming units (CFU) were counted after incubation at the appropriate temperature. Three
241 independent experiments were performed.

242

243 **3. Results**

244 **3.1 Fluorescent labelling of *Lactobacillus* strains with pRCR12 and detection of the mCherry** 245 **protein**

246 The detection of transformants of the *L. plantarum* 90, *L. plantarum* B2 and *L. fermentum* 11.5
247 strains carrying the pRCR12 plasmid was easy, since the colonies containing the plasmid-carrying
248 bacteria showed a pink colour after two days of incubation, which varied from dark to pale

249 depending on the strain (Figure 2). In addition, the fluorescence was still visible after eleven days
250 incubation of the plates (supplementary figure S1), indicating the high stability of the plasmid and
251 the mCherry protein in these hosts.

252 The differences observed between strains correlated with the levels of fluorescence detected in
253 exponential bacterial cultures analyzed by fluorescent microscopy (Figure 2) and spectroscopy (data
254 not shown). The dark pink colour of *L. plantarum* 90/pRCR12 colonies correlated with the bright
255 red colour of the bacterial cells and the highest levels of fluorescence (100 units), whereas for *L.*
256 *plantarum* B2/pRCR12 and *L. fermentum* 11.5/pRCR12 the medium-pale pink colony colour
257 correlated with the less fluorescent dark red appearance of the cells under the fluorescent
258 microscopy and lower levels of fluorescence (52 and 42 units, respectively).

259 These differences could be due to variations of the pRCR12 gene doses in the different hosts.
260 Analysis of the pRCR12 plasmid content in the three transformants is shown in supplementary
261 figure S2. Moreover, other plasmids were detected in *L. plantarum* B2/pRCR12 showing that the
262 pRCR12 system can potentially be used to tag multiplasmid-carrying lactobacilli. DNA extracts
263 containing both plasmidic and chromosomal DNA were used to determine the plasmid copy number
264 in the different hosts. The results gave values of 62 ± 2 , 54 ± 3 and 33 ± 1 pRCR12 plasmid DNA
265 molecules per bacterial genome, in the 90, B2 and 11.5 strains respectively. Thus, these results may
266 explain the differences in the levels of bacterial fluorescence among the three strains.

267 The levels of fluorescence allowed the measurement of mCherry active protein in real time during
268 growth. Thus, fluorescence as well as the optical density of cultures grown in liquid medium were
269 analyzed during the different phases of growth and the results are depicted in Figure 3. During the
270 exponential phase of growth, in all three recombinant strains the fluorescence increased with
271 incubation time, though they reached different levels of intensity. In *L. plantarum* 90/pRCR12 only
272 a further marked increase was observed during the stationary phase (Figure 3). This result could

273 indicate that mCherry is more stable in this strain. Although *L. fermentum* 11.5/pRCR12 exhibited
274 the lowest fluorescence, it was, nevertheless, clearly detectable, and therefore our findings showed
275 that pRCR12 could serve as a general fluorescent tag of Lactobacilli. Growth curves of non-
276 recombinant strains were compared with those of the corresponding pRCR12-carrier strains and no
277 relevant difference in growth rates were observed (data not shown), indicating that the presence of
278 the pRCR12 plasmid does not interfere with bacterial growth.

279 **3.2 Colonization of zebrafish larvae by probiotic LAB strains**

280 The mCherry labelling allowed the monitoring, with high resolution level, of the *in vivo*
281 colonization ability of LAB strains in the intestinal tract of the zebrafish larvae and detection of
282 their adhesion to enterocytic cells (Figure 4). At 6 hours post infection (hpi), red fluorescence
283 revealed the occurrence of all the strains in the intestinal tract of the larvae. However, in the case of
284 *L. fermentum* 11.5/pRCR12 the intensity of the fluorescence appeared to be noticeably weak at 6
285 hpi, and it was undetectable at 24 hpi (data not shown). By contrast, at the same time periods,
286 abundant red fluorescence was visible in larvae exposed to both *L. plantarum* strains (Figure 5),
287 though differences were detected between them. At 6 hpi larvae exposed to Lp90/pRCR12 emitted
288 a statistically significant higher percentage of fluorescence than those infected with B2/pRCR12
289 (Figure 6A). Moreover, under the same conditions, less than 5 % of fluorescent larvae were
290 detected after exposure to *L. fermentum* 11.5/pRCR12 (data not shown). This different behaviour
291 could be affected by the dissimilar level of fluorescence previously observed in bacterial cells in the
292 *in vitro* assays (Figures 2 and 3). However, the fluorescent signal was never perceptible at 72 hpi
293 (data not shown).

294 Interestingly, in larvae inoculated with both *L. plantarum* strains, a spatial displacement of bacteria
295 from the medium to the posterior intestinal tract was observed during the time, which suggested a
296 transient colonization by these probiotics (Figure 5 and 6B). In particular, after 6 hpi few larvae

showed red fluorescence in the posterior intestine when they were exposed to either B2/pRCR12 or 90/pRCR12 strains (Figure 6B). This percentage increased after 24 hpi ranging from 20 to 30 % of the total (Figure 6B). In order to confirm that fluorescence was related to active adhesion by probiotic LAB in the intestinal tract, zebrafish larvae were euthanized and the number of viable bacteria at each time of analysis was determined by plate count. Zebrafish gastrointestinal tract retained bacteria of both *L. plantarum* strains in variable amounts (Figure 7). At 6 hpi, 90/pRCR12 viable cells were significantly higher, although the number decreased over time. Conversely, at 24 hpi, B2/pRCR12 exhibited significantly higher CFU than 90/pRCR12. Beyond 48 hpi, the bacterial CFU per larva was very low and most larvae did not show any detectable bacteria (Figure 7). However, the low CFU values do not allow a reliable quantitative analysis of the colonization ability of the investigated strains.

No viable bacterial cells could be detected in larvae infected with *L. fermentum* (data not shown) which could account for the scarce fluorescence shown by the larvae at microscopic analysis, thus substantiating a low adhesive capacity of the *L. fermentum* strain compared to the other Lactobacilli tested.

4. Discussion

In this study we developed a mCherry-based reporter system to evaluate the probiotic potential of LAB strains in a gnotobiotic zebrafish larvae model. Nowadays, fluorescent labelling methods are increasingly being used to obtain real-time and *in vivo* evidence of a wide range of biological phenomena (Chudakov et al. 2005). Such approaches have been already successfully exploited to provide insights in the field of probiotics. In recent years, strains tagged with reporter genes were used to monitor the fate of *Bifidobacterium* species in complex ecosystems like food and faecal microbiota (Landete et al. 2014), or within the mouse gastrointestinal tract (Cronin et al. 2008). However, tracking of fluorescence or luminescence in biological environments is mainly based on

the detection of green fluorescence proteins (GFP) or on luciferase-based systems. Recently, we have developed a family of novel promoter-probe vectors and reporter plasmids carrying out a synthetic mCherry autofluorescent protein optimized to obtain fluorescent LAB (García-Cayuela et al. 2012). This family of plasmids are not functional in Lactobacilli (unpublished results). Therefore, in the present study, after construction of pRCR12, we report for the first time on the expression of the monomeric mCherry protein in *L. plantarum* and *L. fermentum* backgrounds, further supporting that this plasmid provides an easy way for introducing the autofluorescent mCherry proteins in lactobacilli belonging to different species. Moreover, in agreement with previous observations in *Lactococcus lactis* and *Enterococcus faecalis* (García-Cayuela et al. 2012), the expression of mCherry was easily detected by the pink colour of the colony in both lactobacilli species, while real-time fluorescence evolved in parallel to the growth of the *L. plantarum* strains. This indicated that the mCherry reporter could be successfully employed as a strategy to track in real-time the fate of potential probiotic strains within the gut of transparent gnotobiotic zebrafish larvae. Additionally, the combination of mCherry reporter in LAB strains with, for example GFP-expressing bacteria, will allow the study of *in vivo* competition assays.

Recently, the ability of LAB to colonize the zebrafish intestinal tract has been reported from studies mainly focused on the potential beneficial effects of probiotic administration for aquaculture applications (reviewed by Carnevali et al. 2013). However, although strains belonging to several LAB species could be considered good probiotic candidates, most of the studies on zebrafish have used *Lactobacillus rhamnosus* as a reference microorganism (Avella et al. 2012; Maradonna et al. 2013). Therefore, studies using a wide range of probiotic microorganisms in the zebrafish model are lacking and further insights on this topic should be encouraged.

Recently, the use of GFP-producing *Vibrio cholera* cells allowed an easy visualization of the gut infection in zebrafish larvae (Runft et al. 2014). A similar approach was proposed for the first time

345 as an *in vivo* screening system to detect probiotic strains with anti-inflammatory properties (Rieu et
 346 al. 2014). The authors of this study infected zebrafish larvae with a strain of *Lactobacillus casei* and
 347 used GFP-expression to visualize their location in the gut (Rieu et al. 2014).
 348 In an analogous way, in the present work the zebrafish model was assessed by using three different
 349 strains belonging to the *L. plantarum* and *L. fermentum* species. Microorganisms of these species
 350 are commonly found in several fermented foods and often exhibit strain-specific probiotic
 351 properties (Turpin et al. 2012; Ramos et al. 2013). In this study we have advanced the knowledge of
 352 microbial strains that we had previously investigated for their probiotic behaviour (Arena et al.
 353 2014; Caggianiello et al. unpublished results). In particular, *L. plantarum* 90, a potential probiotic
 354 strain from wine origin, was characterized by a ropy phenotype, due to its capacity to produce
 355 exopolysaccharides (EPS) (Caggianiello et al. unpublished results). So far, the probiotic potential of
 356 oenological LAB has been analyzed only in few cases, and the EPS-producing phenotype was one
 357 of the more attractive features (García Ruíz et al. 2014). There is evidence that EPS from LAB can
 358 regulate inflammatory responses in the intestinal lumen (Notararigo et al. 2014), and moreover, EPS
 359 could exert some prebiotic activity (Russo et al. 2012).
 360 The other two strains analyzed in this study are the riboflavin overproducing *L. plantarum* B2 and
 361 *L. fermentum* 11.5 (Arena et al. 2014; Russo et al. 2014). These microorganisms were previously
 362 shown to adhere *in vitro* to human intestinal epithelial cells and to be able to synthesiz
 363 e vitamin B2 in co-culture systems with Caco-2 cells, suggesting that they could contribute to
 364 further increase the riboflavin supply in the gut environment (Arena et al. 2014).
 365 In the aforementioned work, *L. plantarum* B2 and *L. fermentum* 11.5 were both classified as
 366 strongly adhesive strains, when compared to commercial probiotic strains (Arena et al. 2014).
 367 However, in the present work, the *L. fermentum* capability to colonize zebrafish gut was lower

368 compared to *L. plantarum* strains. Indeed, it is presumable that different levels of adhesion are
369 detected between the *in vitro* and *in vivo* binding phenotypes of the same strain (Turpin et al. 2013).
370 Interestingly, both pRCR12-carrying *L. plantarum* strains showed superior abilities than *L.*
371 *fermentum* 11.5/pRCR12 to colonize the intestinal tract of gnotobiotic zebrafish larvae. In previous
372 *in vitro* investigations, *L. plantarum* B2 proved to be more adhesive compared to *L. fermentum* 11.5
373 (Arena et al. 2014). Our *in vivo* results parallel those reported by Turpin et al. (2013) who found a
374 higher abundance of *L. plantarum* than *L. fermentum* strains in the digestive tract of germfree rats
375 feed with a cocktail of probiotic *Lactobacillus* spp.. Similarly, *L. plantarum* was identified as
376 highly-adhesive when zebrafish adults were fed with a probiotic diet supplemented with ten
377 *Lactobacillus* strains (Zhou et al. 2012). Accordingly, here we found a weak red fluorescence at 6
378 hpi only in the terminal gut tract of larvae infected with *L. fermentum* 11.5/pRCR12, whereas *L.*
379 *plantarum* strains were able to adhere strongly to the posterior intestine of larvae after 24 hpi. At the
380 same time, *L. plantarum* B2/pRCR12 seemed to be displaced with time to the distal gut.
381 Thus, the overall fluorescence data suggest that both *L. plantarum* strains share a similar adhesion
382 capacity, yet different strains of the same species may exhibit preferences for different site of
383 adhesion and/or different gut transition kinetics. Nevertheless, the microbial count analysis
384 indicates that *L. plantarum* B2 has the ability to persist longer in zebrafish gut. However, the very
385 low, probably underestimated, CFU values obtained with this approach could bias a quantitative
386 comparative study of the colonization behaviour of the two strains. Therefore, our data support a
387 strain-dependent colonization capability. This distinct behaviour could be due to either an initial
388 greater persistence of *L. plantarum* 90 or a microbial preference to establish in precise sections of
389 the gastrointestinal tract, as previously reported for *Bifidobacterium breve* (Cronin et al. 2008).
390 Based on the *in vivo* experiments carried out in the present study, *L. plantarum* 90 possesses a very
391 good colonization potential, which could be connected to its ability to synthesize EPS. However the

effect of EPS on microbial adhesiveness is controversial, as contrasting results have been obtained by both *in vitro* and *in vivo* studies. Recently, a positive correlation between EPS production and the percentage of binding to Caco-2 cells was reported for strains isolated from cider and wine (Fernández de Palencia et al. 2009; Garai-Ibabe et al. 2010; García Ruíz et al. 2014). In contrast, Nikolic and coauthors (2012) found that three non-ropy derivatives had improved *in vitro* adhesion compared to the parental phenotypes, suggesting that the presence of a surrounding EPS layer could hinder the attachment to different cell lines. Similar, opposing results were also reported when analyzing *in vivo* models. For instance, the inability to permanently colonize the intestine of germ-free mice was attributed to the EPS-producing properties of *Lactobacillus kefiranofaciens* (Chen and Chen 2013). Conversely, Lebeer et al. (2011) found a higher persistence of *Lactobacillus rhamnosus* GG than its isogenic derivative EPS-mutant when using a murine model. Therefore, the trait conferred by the EPS-producing phenotype in relation to gut colonization should be further investigated.

In our system, the expression of mCherry suggested that the LAB cells were metabolically active throughout the intestinal tract. Nonetheless, only very low values of viable cells could be detected by plate count analysis, which, like other culture-dependent methods, may underestimate the quantification of viable bacteria (Davis 2014). Such finding is in agreement with previous reports (Avella et al. 2012; Rieu et al. 2014) and suggests that monitoring fluorescence, rather than direct bacterial enumeration, could be a better approach to estimate the colonization of zebrafish gut larvae. In addition, the reporter system described in this work avoids the need to sacrifice the animal, thus ensuring that experiments are both scientifically and ethically justified (Dothel et al. 2013).

Compared with traditional microbiological (i.e. plate count analysis) or molecular-based (i.e. quantitative PCR) methods, the use of fluorescently labelled LAB provides valuable advantages, as

416 it can not only detect and quantify adhesion to the gut epithelium, but also monitor the transition
417 kinetics along the gastro-intestinal tract and evaluate possible preferential tropisms for gut
418 segments.

419 In conclusion, we have confirmed that the pRCR12 plasmid is a suitable tool to express the
420 fluorescent mCherry protein in different LAB backgrounds, offering a useful alternative to, and
421 complementing the GFP-expressing reporters. We have successfully used this construction to
422 monitor in real time the colonization and persistence of exogenous LAB within the gut of zebrafish
423 larvae, which we regard as a promising model for *in vivo* investigations in the probiotic field. This
424 approach could bring further insights into the complex microbial-host interactions at intestinal level.
425 Finally, our results further support the probiotic potential of three LAB strains of interest to the food
426 industry.

427

428 **5. Acknowledgments**

429 We sincerely thank Dr. César Martín Plágaro (UPV/EHU, Leioa, Spain) for Confocal Microscopy
430 analysis. We thank Dr. Stephen Elson for the critical reading of the manuscript.

431 The work performed in Spain was supported at the Centro de Investigaciones Biológicas by grant
432 AGL2012-40084-C03-01 from the Spanish Ministry of Economics and Competitiveness, and at
433 AZTI-Tecnalia by the “Departamento de Desarrollo Económico y Competitividad Viceconsejería
434 de Agricultura, Pesca y Políticas Alimentarias” (Project FOODBASK3). Iñaki Iturria is recipient of
435 a PhD fellowship from Iñaki Goenaga Foundation.

436 The work performed in Italy was supported by the Italian Ministry for Development in the
437 framework of the “Industria 2015 Bando Nuove Tecnologie per il Made in Italy - Realizzazione di
438 una innovativa pasta alimentare funzionale arricchita di componenti bioattivi e probiotici”, and by

439 MIUR [PON02_00186_2937475] in the framework of the project named “Protocolli innovativi per
440 lo sviluppo di alimenti funzionali” [Pro.Ali.Fun.]”.

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582 **Legend to the figures**

583 **Figure 1. Physical map of pRCR12.** The plasmid carries: the *mrfp* gene encoding the mCherry
584 protein under the control of the Px promotor of *S. pneumoniae*; the *cat* gene, which confers
585 chloramphenicol resistance and it contains the replicon of plasmid pSH71 from *L. lactis*.

586 **Figure 2. Detection of mCherry fluorescence in colonies and cells of the indicated LAB.**
587 Images of the bacterial colonies grown in MRS agar plates and of exponential-grown bacteria under
588 exposure to a fluorescence microscope are depicted.

589 **Figure 3. Spectrophotometric detection of LAB tagged with pRCR12.** Optical density (OD₆₀₀
590 nm) and mCherry fluorescence levels of *L. plantarum* 90/pRCR12 (A), *L. plantarum* B2/pRCR12
591 (B), and *L. fermentum* 11.5/pRCR12 (C) cultures were monitored in real time during 17 hours.

592 **Figure 4. Detection of binding of *L. plantarum* 90/pRCR12 to zebrafish larvae enterocytes.**
593 Images were captured at 48 hpi using a confocal microscope. Full and dashed white arrows mark
594 the localization of LAB and enterocytes, respectively.

595 **Figure 5. Detection of intestinal distribution of Lactobacilli tagged with pRCR12.** Zebrafish
596 larvae infected with either *L. plantarum* 90/pRCR12 or *L. plantarum* B2/pRCR12 observed under a
597 fluorescence stereomicroscope at 6, 24 and 48 hpi. White arrows mark the localization of LAB
598 strains in the medium (a) or posterior (b) intestinal tract.

599 **Figure 6. Quantification of *L. plantarum* strain prevalence in zebrafish larvae digestive tract**
600 **by mCherry fluorescence measurement.** The percentage of the total zebrafish larvae presenting
601 fluorescence (A) and occurrence of the fluorescence in the medium (filled bars) or in the posterior
602 (dotted bars) intestine (B) at 6 and 24 hpi with *L. plantarum* 90/pRCR12 (white bars) or *L.*
603 *plantarum* B2/pRCR12 (black bars) is depicted. Each bar represents the mean of three replicates of
604 15 larvae each. Error bars indicate the standard error of the mean. Student t-test analysis was carried
605 out and differences were considered statistically significant at $p < 0.05$.

606 **Figure 7. Quantification of *L. plantarum* strains prevalence in zebrafish larvae digestive tract**
607 **by plate count.** Colonization of zebrafish larvae intestines by *L. plantarum* 90/pRCR12 (white
608 bars) and *L. plantarum* B2/pRCR12 (black bars) was determined by plate count at 6, 24, 48, 72 hpi.
609 Each bar represents the mean of three replicates of 5 larvae each. Error bars indicate the standard
610 error of the mean. Student t-test analysis was carried out and differences were considered
611 statistically significant at $p < 0.05$.

612 **Figure S1.** Colonies of Lp90, *L. plantarum* B2 and *L. fermentum* 11.5 carrying pRCR12 on MRS
613 agar plates after 2 and 11 days of incubation at 37 °C.

614 **Figure S2.** Analysis of pRCR12 plasmid. Total DNA extracts from the indicated strains carrying
615 the plasmid were analyzed.

616 **Figure 1**

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628 **Figure 2**

Figure 2

641 **Figure 3**

Figure 3

652 **Figure 4.**

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661 **Figure 5.**

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L. plantarum 90/pRCR12

L. plantarum B2/pRCR12

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6 hpi

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24 hpi

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48 hpi

685 **Figure 6.**

686 **A**

688 **B**

693 **Figure 7**

710 **Figure S1**

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