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Growth phase of orally administered *Lactobacillus* strains differentially affects IgG1/IgG2a ratio for soluble antigens: implications for vaccine development

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Abstract

Lactobacillus strains with probiotic activity are major constituents of numerous common food products. Due to their 'generally regarded as safe'-status (GRAS-status), *Lactobacillus* strains can also be genetically engineered for use in oral immunotherapeutic applications, such as vaccination and T lymphocyte tolerance induction in autoimmune disease.

In the current study, we demonstrate that the growth phase of orally administered individual *Lactobacillus* strains can differentially affect antigen-specific antibody subclasses IgG1 and IgG2a, which might reflect skewing of systemic activity of T helper cell type 2 (Th2) and T helper cell type 1 (Th1) pathways, respectively. Mice were orally fed different wild type *Lactobacillus* strains in log phase or stationary phase and immunized intraperitoneally with a T-cell dependent protein antigen. Sera were evaluated for the ratio of antigen-specific IgG1 and IgG2a antibodies. Stationary *Lactobacillus murines* and *Lactobacillus casei* cultures, but not two other *Lactobacillus* strains, evoked significantly higher IgG1/IgG2a ratios than log phase cultures, possibly relating to increased activity of the Th2-pathway. Despite normal variation in antibody responses against TNP–CGG among individual mice, a high correlation was found between the IgG1 and IgG2a responses of mice within experimental groups. This differential antibody response is likely due to growth phase-dependent differences in bacterial cell composition.

Since *Lactobacillus* growth phase dependent skewing of antibody responses possibly reflecting T-cell pathways can inadvertently affect allergic and (auto)-immune responses, the current findings strongly caution against unidimensional views on the oral administration of individual *Lactobacillus* strains for probiotic or immunotherapeutic purposes, but also suggest additional possibilities for immune modulation.
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Keywords: Th1–Th2; Antibodies; Immunomodulators

1. Introduction

The genus *Lactobacillus* belongs to the family of Lactobacteriaceae, also known as lactic acid bacteria, a group of microorganisms that have been used for centuries in bio-processing and preservation of food and feed. Some fermented dairy products containing *Lactobacillus* species are believed to have certain health promoting properties for humans, including enhancement of immune responses (adjuvanticity) and anti-carcinogenic activity. As lactobacilli are commensals of the gut and are generally regarded as safe (GRAS-status), lactobacilli are good candidates for use as production and delivery hosts of heterologous proteins in food but also health care products. Genetically engineered lactobacilli expressing bacterial or viral proteins on the cell surface can potentially be used as oral vaccines [1,2]. On the other hand, *Lactobacillus* recombinants secreting tolerogenic autoantigens can be used to induce systemic peripheral T-cell tolerance by oral administration for treatment of autoimmune disease [1].

Abbreviations: GRAS, generally regarded as safe; DTH, delayed type hypersensitivity; TNP, trinitrophenyl; CGG, chicken gamma globulin; PVC, polyvinyl chloride; a.u., arbitrary unit; EAE, experimental autoimmune encephalomyelitis; DC, dendritic cell

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Clearly, there is accumulating evidence that lactobacilli possess properties that are beneficial in a diverse range of diseases, including infections, allergy and cancer [3–8]. However, due to the diversity of species within the *Lactobacillus* genus not each *Lactobacillus* strain possesses similar health stimulating properties. For instance, it has been shown that *Lactobacillus* strains differ in their ability to enhance the humoral immune response (adjuvant activity) and that they induce distinct cytokine profiles in the gut after oral administration [9,10]. This implies that strain selection is a crucial issue for the use of wild type *Lactobacillus* strains in food products, but also when recombinant lactobacilli are to be used to promote different types of immune responses such as vaccination, T-cell tolerance induction and treatment of allergy.

Most immune responses are centrally regulated by the activity of two functionally polarized T helper cell types, Th1 versus Th2 cells (reviewed in [11]). Th1 cells producing IFN-γ and IL-12 promote cellular immune responses including macrophage activation, delayed type hypersensitivity (DTH) and cytotoxicity. Th1 cells positively contribute to the humoral (antibody) response to a limited extent by supporting the production of the IgG2a antibody subclass, but they inhibit production of several other subclasses like IgG1. Although the Th1 pathway is crucial for protection against many pathogens, it may also be the predominating type of response in chronic inflammatory autoimmune diseases like multiple sclerosis and insulin-dependent diabetes mellitus [11–13].

Th2 cells produce IL-4, IL-5 and IL-10, which are generally regarded to be anti-inflammatory. These cytokines are involved in stimulating strong antibody production by B cells (e.g. IgG1, IgM and IgE), stimulation of eosinophils and mast cells, as well as deactivation of macrophages. Dominant Th2 responses may contribute to diseases such as atopic allergy. In mice, production of IgG1 versus IgG2a is widely interpreted as a reflection of differential Th2–Th1 reactivity. Since differential functional capabilities of IgG1 versus IgG2a have been documented extensively, for instance with respect to complement fixation, the IgG1/IgG2a ratio can be regarded as an indirect reflection of immune function in vivo.

A multitude of approaches to skew T helper cell pathways to treat human diseases is under intense investigation [14]. When oral bio-therapy with wild type *Lactobacillus* strains or genetically engineered recombinants [1,15,16] is considered, the way these strains impinge on T helper pathways is an important factor determining successful application. Therefore, the aim of the present study was to establish whether orally administered individual *Lactobacillus* strains can differentially affect IgG1 (Th2) versus IgG2a (Th1) antibody responses against systemically administered exogenous protein antigen, possibly reflecting T helper cell pathways and whether such skewing is dependent on the growth phase of the lactobacilli.

2. Materials and methods

2.1. Animals and Lactobacillus strains

Female SJL/J mice (Erasmus University Rotterdam, The Netherlands) were kept under filtertop hoods in a DII facility with free access to mouse chow and acidified water (pH 2.8). Experiments were performed according to regulations in the Dutch law on animal experimentation.

The *Lactobacillus* strains *L. reuteri ML1* and *L. murines CNRZ* were originally isolated from mouse. The strains *L. casei ATCC 393* and *L. plantarum NCIB 8826* were originally isolated, respectively, from cheese and human saliva.

2.2. Culturing of Lactobacillus strains

All *Lactobacillus* strains were cultured overnight, in 5 ml MRS broth followed by 15 ml MRS broth (Difco, Detroit, MI), before inoculation in 500 ml MRS broth (1:50). The cultures were grown to late log phase (6 h) or stationary phase (16 h). The cells were harvested, washed twice with PBS and once with 0.2 M $NaHCO_3$. This extensive washing was performed to ensure no secreted *Lactobacillus* products were present in the suspension. Before use the cells were resuspended in 0.2 M $NaHCO_3$. From this suspension a sample was taken for plating on MRS broth plates for the calculation of the number of colony forming units (CFU) as well as to rule out contamination.

2.3. Oral administration of Lactobacillus strains and immunization schedule

To investigate the effect of orally administered lactobacilli on the humoral immune response, mice orally received wild type lactobacilli, prepared as described earlier, on four consecutive days. Each animal received approximately 10^{10} CFU per intra-gastric administration. On the first day, the mice were also immunized intraperitoneally with 25 μg of the hapten trinitrophenyl (TNP) conjugated to the thymus dependent antigen chicken gamma globulin (CGG; Sigma, La Jolla, CA). One control group was immunized with TNP–CGG in a water-in-oil adjuvant, specol [17], which resembles Freund's incomplete adjuvant. From day 49 onwards, oral administration of lactobacilli as well as intra-peritoneal immunization with TNP–CGG was repeated as above. This day was chosen as titers had declined to below 50% of maximal values. Blood samples were taken before the start of the experiment and every 7 days.

2.4. Semi-quantitative ELISA

Serum titers of CGG and TNP specific antibodies after immunization with TNP–CGG were determined by ELISA. Polyvinyl chloride (PVC) microtiter plates (Titertek, Flow Laboratories, Irvine, Scotland) were coated with CGG

(5 μg/ml, 50 μl/well) or TNP–BSA (5 μg/ml, 50 μl/well) overnight at 4 °C. Non-specific antibody binding was blocked by incubation with 0.2% gelatin in PBS (50 μl/well) for 1 h at 25 °C. Subsequently the plates were incubated for 1 h at 25 °C with dilutions of TNP–CGG induced antisera and pre-immune sera to correct for background reactivity. For the detection of IgG antibodies specific for CGG or TNP, alkaline phosphatase-labelled goat anti-mouse IgG (KPL, Gaithersburg, MD) was used. For the detection of CGG or TNP specific IgG1 or IgG2a antibodies, rabbit anti-mouse IgG1 or rabbit anti-mouse IgG2a antibodies (ICN Immunobiologicals, Costa Mesa, CA) were used, respectively, followed by 1 h incubation with alkaline phosphatase-labelled swine anti-rabbit Ig antibodies (Dako A/S, Glostrup, Denmark). After addition of the substrate *para*-nitrophenyl phosphate, the absorbance was read at 405 nm. An IgG1 monoclonal antibody directed against CGG was used as reference for detection of IgG and IgG1 CGG specific antibodies on each ELISA-plate. A polyclonal mouse serum containing high levels of IgG2a antibodies specific for CGG was used as reference in the ELISAs to detect IgG2a specific antibodies against CGG. The reference used in all TNP-specific ELISAs was a polyclonal mouse serum with high levels of IgG1 and IgG2a TNP-specific antibodies.

Relative concentrations of IgG1, IgG2a and total IgG were calculated after subtraction of the absorbance of pre-immune sera at the corresponding dilutions. The linear part of the reference curve was used to perform linear regression. Only those measurements of the test sera falling within the same absorbance range as reference samples used for regression (with comparable slope), were used for the calculation of the relative concentrations of IgG1, IgG2a and IgG in arbitrary units (a.u.).

To detect antibody responses against *Lactobacillus murines* after oral administration, PVC plates were coated with fragmented *L. murines* (50 ng/ml, 50 μl/well). The fragments were obtained by sonification of cells harvested after overnight growth in MRS broth. The following incubation steps and development were performed as described for total IgG. Serum obtained after intra-peritoneal immunization of mice with *L. casei* in adjuvant (Difco's complete adjuvant, Difco) was used as reference.

2.5. Statistical analysis

A single factor ANOVA was used to analyze the data. As the groups of mice were too large to be held in one cage, it was calculated whether there were differences between cages holding mice of the same group. Since this was not the case, the *t*-alfa for students *t*-test could be used to calculate the least significant differences instead of the *F*-alfa of the *F*-test. When $P < 0.05$ the difference was interpreted as significant. This approach was used to compare the total IgG antibody responses and the IgG1/IgG2a responses of the groups on 1 day (Figs. 1 and 2). When the IgG1/IgG2a ratios were compared over time (7, 14 and 21 days after the second immunization), linear regression was performed on the data of each individual animal. The same procedure as used for the single data points was applied to the regression lines (Fig. 4). Differences between the intercepts indicate different IgG1/IgG2a ratios. Distinct slopes indicate difference in change of the ratios over time. Linear regression was performed to calculate the correlation lines in Fig. 3.

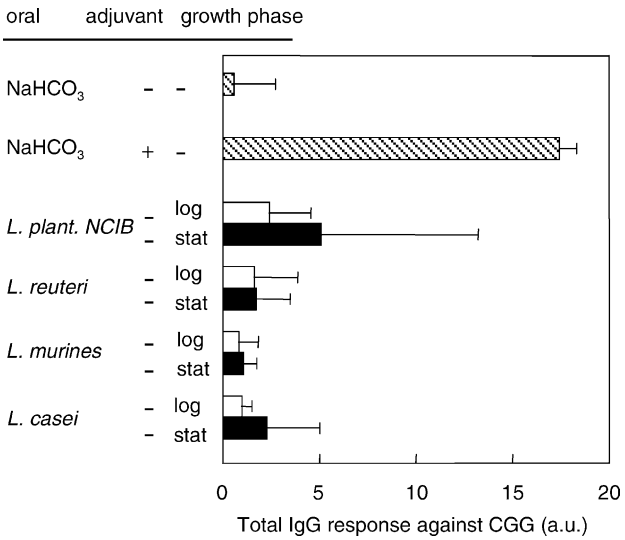

Fig. 1. Four wild type *Lactobacillus* strains do not enhance systemic antibody responses. Total IgG antibody responses against CGG were determined in sera of mice immunized i.p. with TNP–CGG in PBS ($n = 5$). One control group was immunized with TNP–CGG in adjuvant. The mice received wild type lactobacilli in $NaHCO_3$ orally on four consecutive days. The lactobacilli were grown to log phase (white bars) or stationary phase (black bars). The control groups received $NaHCO_3$ buffer only (hatched bars). The immunization with TNP–CGG as well as the oral administration procedure was repeated from day 49 onwards. Results (±S.E.M.) obtained with sera collected 14 days after this second immunization are shown. A reference curve was used to calculate the arbitrary units; stat, stationary.

3. Results and discussion

3.1. Four wild type Lactobacillus strains do not enhance systemic antibody responses

In order to investigate whether different *Lactobacillus* strains have different intrinsic adjuvanticity (defined as the capability to non-specifically enhance the antibody response), four wild type *Lactobacillus* strains were orally administered to SJL/J mice ($n = 5$). SJL/J mice are biased towards the Th1 pathway and are susceptible to induction of experimental autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis. The bacteria were grown to log phase (6 h) or stationary phase (16 h) to investigate whether the intrinsic adjuvanticity is growth phase dependent. The mice received

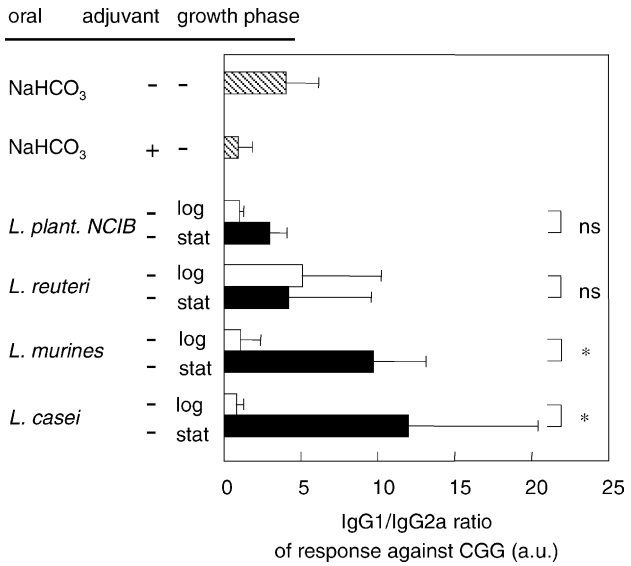

Fig. 2. Two wild type *Lactobacillus* strains skew the IgG1/IgG2a ratio dependent on growth phase. Mice ($n = 5$) were immunized i.p. with TNP–CGG in PBS and fed with wild type *Lactobacillus* strains in log (white bars) or stationary phase (black bars). The control groups orally received $NaHCO_3$ only (hatched bars) and were immunized i.p. with TNP–CGG in PBS or adjuvant. The IgG1 and IgG2a responses were calculated in arbitrary units with the use of reference curves. The ratio between IgG1 and IgG2a antibodies specific for CGG was calculated for sera obtained 14 days after the second immunization with TNP–CGG (±S.E.M.). Significance of differences observed between groups of mice fed log or stationary phase cultures of the same *Lactobacillus* strain is indicated; stat, stationary.

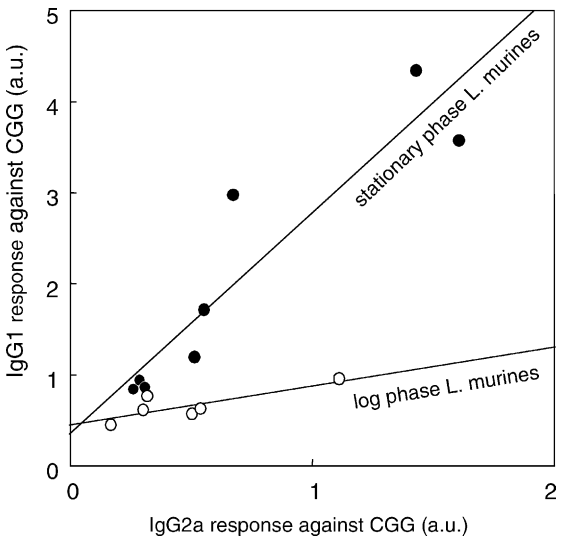

Fig. 3. *L. murines* of different growth phases induce distinct but constant IgG1/IgG2a ratio's. Mice ($n = 8$) were fed *L. murines* in stationary or log phase. Sera obtained 21 days after the second immunization with TNP–CGG were tested for IgG1 and IgG2a antibody responses against CGG. IgG1 and IgG2a responses are indicated in arbitrary units which were calculated with the use of reference curves. The IgG2a response of two animals in the group fed log phase *L. murines* was too low to be calculated. These mice were excluded from the analysis to prevent undue further emphasis on the existing clear difference between the groups. Each circle represents an individual animal. Mice fed log phase *L. murines* are indicated by (○) and mice fed stationary phase *L. murines* are indicated by (●). The correlation lines were calculated by linear regression. The variance is 0.67 for the group fed log phase *L. murines* and 0.85 for the group fed stationary phase cultures. $R = 0.43$ for the group fed log phase cultures and $R = 2.43$ for the group fed stationary phase cultures.

one of the wild type *Lactobacillus* strains, *L. plantarum NCIB*, *L. reuteri*, *L. murines* or *L. casei* in $NaHCO_3$ orally on four consecutive days. The control groups received $NaHCO_3$ only. On the first day, the mice were also immunized i.p. with TNP–CGG in PBS. One control group was immunized with TNP–CGG in adjuvant. Total IgG antibody responses against CGG were determined in sera collected 14 days after the second immunization (Fig. 1). None of the tested *Lactobacillus* strains exhibited intrinsic adjuvanticity in SJL mice, as no significant differences between groups fed *Lactobacillus* strains and the control group immunized with TNP–CGG in PBS and fed with $NaHCO_3$ only were seen. These data are consistent with results obtained with log phase *Lactobacillus* strains in the Th2-biased BALB/c mouse strain, except for *L. reuteri* [9]. This strain did enhance the antibody response against CGG in BALB/c mice. In addition, no significant differences between log and stationary phases lactobacilli fed groups were present in SJL/J mice, indicating that in this Th1-biased mouse strain, the growth phase of these *Lactobacillus* species does not affect adjuvant activity. As expected, the control group immunized with TNP–CGG in adjuvant showed a significant and strong increase in CGG-specific antibody levels when compared to all other groups ($P < 0.01$). Total IgG responses against TNP were lower but comparable to the anti-CGG responses.

3.2. Two wild type Lactobacillus strains skew the IgG1/IgG2a ratio dependent on growth phase

Although no significant differences occurred in the total IgG response of mice fed log or stationary phase cultures of the same *Lactobacillus* strain, the antibody response might be qualitatively different. For mice, it is generally accepted that the IgG1 response reflects helper activity of Th2 CD4+ T-cells, where IgG2a results from Th1-activity. Therefore, the IgG1 and IgG2a responses against CGG were calculated for sera obtained 14 days after the second immunization and were expressed as the IgG1/IgG2a ratio (Fig. 2). Oral administration of a stationary phase culture of *L. casei* or *L. murines* lead to a significantly higher IgG1/IgG2a ratio than log phase bacteria of the same strain ($P < 0.01$). Although *L. plantarum NCIB* induced a similar pattern of IgG1/IgG2a ratios, the difference did not reach significance. *L. reuteri* did not show a difference in IgG1/IgG2a ratio between log and stationary phases cultures. Because of the differences found between *Lactobacillus* strains, it can be concluded that this isotype skewing is growth phase-dependent as well as bacterial strain-dependent. Although the anti-TNP IgG2a responses in many cases were too low to properly calculate

IgG1/IgG2a ratios, the ratios that were calculated showed a similar trend as the ratios for anti-CGG (data not shown).

3.3. No systemic antibody response against L. murines after oral administration

From the previous experiments it was clear that some *Lactobacillus* strains are able to skew the IgG1/IgG2a response dependent on their growth phase. In addition to *L. casei*, *L. murines* showed a growth phase-dependent difference in IgG1/IgG2a ratio. *L. murines* was selected for further investigation. One of the rationales to use lactobacilli for oral vaccination or other therapeutical purposes is the fact that they are commensals of the human gut [18,19] and are generally regarded as safe (GRAS-status). In addition, they should be non-immunogenic reflected in an inability to evoke antibody responses against themselves. To evaluate this in the current experimental setting, a similar experimental design as in Figs. 1 and 2 was used. Total IgG and IgM responses of SJL/J mice ($n = 8$) against *L. murines* were determined by ELISA in pooled sera obtained 21 days after the second intra-peritoneal immunization with TNP–CGG and oral administration of *L. murines*. As expected, the treatment protocol did not induce IgG responses against *L. murines* itself. For *L. murines* an absorbance of 0.26 was measured for log phase cultures and 0.22 for stationary cultures, versus absorbances of 0.25 for normal mouse serum and 0.34 for mice which received $NaHCO_3$ only. No IgM response could be detected either (data not shown). In contrast, when separate mice were immunized intraperitoneally with *L. murines* in adjuvant as a positive control group, high IgG responses against *L. murines* itself occurred (OD (405 nm) = 1.07). These data confirm that oral administration of *L. murines* does not induce antibody responses against the bacteria, and imply that growth phase-dependent skewing of the TNP–CGG specific IgG1/IgG2a ratio is not related to an antibody response against the bacteria.

3.4. L. murines of different growth phases induce distinct but constant IgG1/IgG2a ratios

The skewing of IgG1/IgG2a ratios as depicted in Figs. 1 and 2 was further confirmed in the experiment described above. The total IgG responses against CGG and TNP were similar to the data presented in Fig. 1, although the total IgG response against CGG after oral administration of *L. murines* in stationary phase to mice ($n = 8$) was more pronounced (data not shown). From the previous experiments (Figs. 1 and 2) it was apparent that the IgG1/IgG2a ratio is independent of the level of the total IgG response. This raised the question whether the IgG1/IgG2a ratio is also independent of the level of the IgG1 and IgG2a response per animal, i.e. whether the ratio is constant. Therefore, in Fig. 3, the IgG1 response of individual animals is depicted against the IgG2a response of the same animal, with each circle representing an individual animal. As comparable observations were made

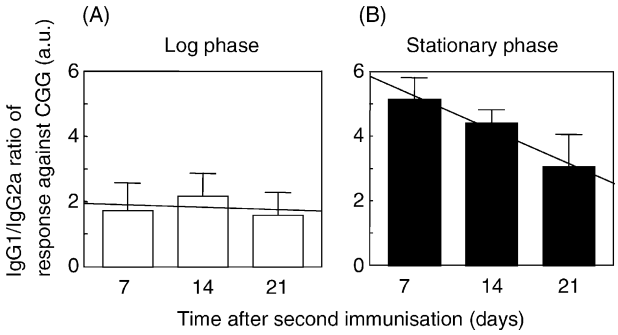

Fig. 4. IgG subclass skewing by stationary phase *L. murines* decreases but persists over time. The ratio of IgG1 and IgG2a antibodies specific for CGG was calculated for sera obtained 7, 14 and 21 days after the second immunization. (A) IgG1/IgG2a ratio of response against CGG in mice fed *L. murines* in log phase (±S.E.M.). (B) IgG1/IgG2a ratio of response against CGG in mice fed *L. murines* in stationary phase (±S.E.M.). The IgG1/IgG2a ratio decreased significantly ($P < 0.05$) over time when *L. murines* in stationary phase was fed ($R = -1.04$), in contrast to a similar IgG1/IgG2a ratio over time when *L. murines* in log phase was fed ($R = -0.07$).

for time points 7, 14 and 21 days, only data are shown from groups which received *L. murines* in log or stationary phases 21 days after the second immunization. Two conclusions can be drawn from this figure: first, there is a strong correlation between the IgG1 and IgG2a response of each individual animal within an experimental group (variance = 0.67 for the group fed log phase cultures and variance = 0.85 for the group fed stationary phase cultures), despite considerable differences in IgG1 and IgG2a responses between the animals. This strong correlation indicates that the IgG1/IgG2a ratio of individual animals within an experimental group is highly comparable. This was demonstrated not only for the groups fed *L. murines* but also for the control groups fed buffer only. Second, the different slopes of the correlation lines of the groups fed log phase ($R = 0.43$) or stationary phase ($R = 2.43$) *L. murines* indicate that the IgG1/IgG2a ratios between those groups are different (see Figs. 2 and 4) confirming that the growth phase of orally administered lactobacilli differentially affects the antibody response. This is functionally relevant with respect to the differential effector functions of IgG1 versus IgG2a Fc-moieties (e.g. complement fixation).

3.5. IgG subclass skewing by stationary phase L. murines decreases but persists over time

In order to investigate whether the antigen specific IgG1/IgG2a ratios persist over time, the IgG1/IgG2a ratio was calculated for sera obtained at 7, 14 and 21 days after the booster immunization. When the ratios were compared over time, the group fed *L. murines* in log phase (Fig. 4(A)) showed a significantly lower IgG1/IgG2a ratio than the stationary phase fed group (Fig. 4(B); intercept difference with $P < 0.05$). This is consistent with the data presented in Fig. 2. Furthermore, the IgG1/IgG2a ratio decreased

significantly with time when *L. murines* in stationary phase were administered ($R = -1.04$), in contrast to a comparable IgG1/IgG2a ratio over time when *L. murines* in log phase was given ($R = -0.07$; slope difference with $P < 0.05$). This change in IgG1/IgG2a ratio over time was not seen in the control groups ($R = 0.06$ for the buffer fed group and $R = 0.02$ for the group immunized with TNP–CGG in adjuvant).

3.6. Perspectives

This study shows that orally administered individual *Lactobacillus* strains are able to differentially affect IgG1 versus IgG2a antibody responses against a thymus dependent antigen, dependent on the *Lactobacillus* growth phase. Stationary phase cultures showed increased production of IgG1, which was evident even while the Th1-biased SJL/J mouse strain was used. The most likely explanation for these findings is the occurrence of growth phase-dependent differences in bacterial cell wall composition, for which there is ample prior evidence, including proinflammatory compounds such as peptidoglycan and teichoic acids, inducing distinct cytokine patterns [20–24]. Also factors involved in bacterial adhesion and colonization might play a role [25]. Another not mutually exclusive explanation is that log phase versus stationary lactobacilli differentially affect dendritic cell populations (DC1 versus DC2), which govern directional activation and polarization of T-cell subsets during antigen presentation [26]. This possibility is supported by the recent findings that *Lactobacillus* strains can differentially affect cytokine and MHC-II expression in DC [27].

Growth phase dependent skewing of T helper cell pathways has important consequences for industrial application of *Lactobacillus* strains, as skewing may affect desired probiotic activities of food products. With respect to immunotherapeutic applications, including genetically engineered lactobacilli, our data caution that oral administration may inadvertently promote autoimmune or allergic reactions. However, this property of some *Lactobacillus* strains may also offer promising opportunities for immune modulation. Oral vaccination strategies [28,29] may exploit skewing properties to enhance T helper cell pathways dependent on the desired protective response, for example, induction of cell mediated immunity versus antibody production, or preferential induction of specific functional IgG subclasses (e.g. differential complement fixation by IgG1 versus IgG2a). Oral *Lactobacillus* administration strategies for peripheral T-cell tolerance induction as a treatment of autoimmune disease [1,14] may benefit from skewing towards Th2 responses, whereas reduction of Th2 activity is required in treatment of allergy. In a study submitted for publication, we extend these findings by showing that *Lactobacillus* recombinants expressing myelin epitopes can positively modulate T-cell reactivity in a disease model, using Th1-mediated experimental autoimmune encephalomyelitis (EAE).

The current findings strongly emphasize the need for rational *Lactobacillus* strain selection and detailed evaluation prior to application in food or health care products, and imply that the bacterial growth phase is a crucial parameter allowing additional manipulation of immune responses by oral administration of lactobacilli.

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