Running headline: Growth improvement of *Lactobacillus plantarum* by catechin

Improvement of the fermentation performance of *Lactobacillus plantarum* by the flavanol catechin is uncoupled from its degradation

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ABSTRACT

Aims: To determine the influence of the flavanol catechin on key metabolic traits for the fermentation performance of *Lactobacillus plantarum* strain RM71 in different media, and to evaluate the ability of this strain to catabolize catechin.

Methods and Results: Growth monitoring and time course of sugar consumption data tracking in chemically defined medium (CDM), revealed that growth of *Lact. plantarum* strain RM71 upon catechin was characterized by a noticeable shorter lag period, outcome of earlier sugar consumption and lactic acid production courses. Catechin gave rise to higher cell densities compared to controls due to an increased extension of sugar utilization. Fermentation of media relevant for practical fermentation processes with *Lact. plantarum* strain RM71 showed that catechin sped up malic acid decarboxylation, which besides quicker and extended consumption of several sugars, resulted in faster and higher lactic acid production and growth. Spectrophotometric evaluation of catechin by HPLC-DAD and the lack of catechin concentration-dependent effects, showed that the observed stimulations were uncoupled from catechin catabolism by *Lact. plantarum*.

Conclusions: The flavanol catechin stimulated the growth of *Lact. plantarum* strain RM71 by promoting quicker sugar consumption, increasing the extension of sugar utilization and stimulating malic acid decarboxylation. These stimulations are uncoupled from catechin catabolism since *Lact. plantarum* did not catabolize it during fermentation.

Significance and Impact of the Study: This study, for the first time, examined the influence of the flavanol catechin on the fermentation performance of a *Lact. plantarum*
strain in several media under different fermentation conditions. The information could be relevant to control the production and obtain high-quality food products fermented by this microorganism.

INTRODUCTION

The flavanols are a subclass of flavonoids widespread in plant foods. Free or as basic unit of proanthocyanidins, catechin is one of the most common flavanols in the diet and therefore part of our polyphenol intake. Catechin is an effective scavenger of reactive oxygen species and plays a role to protect against degenerative diseases (Crespy and Williamson 2004). Before increasing the serum antioxidant capacity, catechin, either on food or in the GI tract, is in contact with autochthonous microbiota. The knowledge on this interaction is important since this flavanol could influence the fermentation performance and adaptation of these microorganisms to their respective environmental niches. On the other hand the microbial fermentation of catechin could reduce the dietary intake of this flavanol.

As regards to the interaction between food microorganisms and catechin, studies so far have mainly focussed on the lactic acid bacteria *Oenococcus oeni* and *Lactobacillus hilgardii* from wine, a beverage that is recognized as an important source of catechin for human nutrition (Amarowicz et al. 2009). The effect of catechin on the growth of these wine lactic acid bacteria seems to be ambiguous. Thus it was reported that catechin stimulated growth of *Lact. hilgardii 5w* (Alberto et al. 2001), specially in media containing tomato juice which is a source of polyphenols (Hussein et al. 2009), and suggested that catechin consumption caused this effect. In apparent contrast, and by using a similar medium, it was recently described that catechin did not significantly
affect the growth of a different strain, *Lact. hilgardii* 5, suggesting that the ability to
metabolize flavanols could be strain dependent (Figueiredo *et al.* 2008). In the same
work it was reported that, similarly to *Lact. hilgardii*, catechin did not significantly
affect the growth of *Oenococcus oeni* strain VF; however by using a different medium,
Reguant *et al.* (2000) described that though not metabolized, catechin promoted growth
as well as malolactic fermentation in *Oenococcus oeni* strain CECT 4100.

Among lactic acid bacteria *Lactobacillus plantarum* was chosen for the present
study because it shows ability to adapt to several catechin-containing environmental
niches. In particular *Lact. plantarum* is involved in the fermentation of plant foods that
according to Amarowicz *et al.* (2009), are the most important sources of catechins for
human nutrition: tea leaves (Okada *et al.* 1996a, 1996b), which is the most important
source in many countries, grapes and wine (Rodas *et al.* 2005) as well as cocoa and
chocolate (Kostinek *et al.* 2008). Furthermore *Lact. plantarum*, besides its successful
adaptation to these plant niches, persist and survive in the human gastrointestinal tract
(Adlerberth *et al.* 1996), where it also meets with diet flavanols. Regarding *Lact.
plantarum* as a representative starter of catechin-containing food plants, the main goal
of this work was to study for first time the interaction between this bacterium and the
flavanol catechin. On one hand the ability of *Lact. plantarum* to metabolize catechin
during growth was evaluated as it could provide primary insight on potential flavanol
loss in either the plant material or in the GI tract. For this goal a chemically defined
medium (CDM) with catechin as the sole flavanol in the formulation was used. On the
other hand it was studied how catechin affected growth, fermentation of several sugars,
utilization of malic acid and lactic acid production, which are common and essential
traits for the successful fermentation performance of *Lact. plantarum* in several
ecological niches. This knowledge could be important to obtain high-quality food
products fermented by this microorganism and to be valuable to understand the survival and persistence of this microorganism in the GI tract. For this purpose the influence of catechin on the fermentation performance of *Lact. plantarum* was evaluated in CDM and in media relevant for practical fermentation processes.

**MATERIAL AND METHODS**

*Bacterial strain and catechin stock solutions*

*Lactobacillus plantarum* RM71 was originally isolated from red wine at the Instituto de Fermentaciones Industriales (CSIC) (Moreno-Arribas *et al.* 2003) and used through this study. (+)-Catechin (from now catechin) was obtained from Fluka (Buchs, Switzerland); purity was ≥ 98%. A 5% catechin stock solution was prepared in ethanol. Appropriate dilutions were prepared to adjust catechin at final concentrations of 100 or 200 mg l⁻¹ (final concentration), or omitted as indicated.

*Preparation of chemically defined medium. Setting up, inoculation and incubation of microtiter plates*

The chemically defined medium (CDM), previously reported to support the growth of *Lact. plantarum* WCFS1 strain, was prepared as described (Teusink *et al.* 2005), and used for bacterial growth. Briefly CDM contains K₂HPO₄ (1 g l⁻¹), KH₂PO₄ (5 g l⁻¹), sodium acetate (1 g l⁻¹), ammonium citrate (0.6 g l⁻¹), ascorbic acid (0.5 g l⁻¹)
and different quantities of several vitamins, nucleotides, and 18 amino acids. Vitamins, nucleotides and amino acids were prepared separately as stock solutions and frozen at -40 °C before thawed prior to use. Sodium acetate (1 g l⁻¹) and ammonium citrate (0.6 g l⁻¹), which are present in the original CDM formulation, were added or omitted as indicated. The different CDM formulations, i.e. lacking or including citrate and/or acetate, were adjusted to pH 5.5 or 6.5 with 5M NaOH, as required, and then filter sterilized (0.22 µm, pore size). The media was supplemented with 0.5 % galactose instead of glucose as growth sugar and catechin added at 100 or 200 mg l⁻¹ (final concentration).

*Lactobacillus plantarum* RM71 colonies from MRS plates (de Man et al. 1960) were inoculated into fresh, complete CDM medium (3 ml) containing 1% glucose. After 24 h incubation at 30º C, 1 ml of fully grown cells was centrifuged and washed three times with saline solution (0.85% NaCl). Stock CDM solutions containing catechin, ethanol, sodium acetate and/or ammonium citrate or none of these acids at the desired concentrations were aseptically prepared. Galactose and the bacteria were added to the stock solutions, just prior to the incubation step. The inoculum size was 1 % (v/v).

Then, volumes (100 µl each) of the inoculated stock CDM solutions were dispensed on sterile 96-well microtiter plates with lid (Falcon Cajal, Madrid, Spain) and incubated at 30 ºC. Two types of controls were provided: control wells containing uninoculated media (to survey contamination and potential OD₆₀₀ changes due to catechin degradation or precipitation) and inoculated control wells lacking catechin.

**Growth experiments and data handling**
In the experiments carried out in CDM, growth curves were recorded at 30°C by measuring the optical density (OD$_{600}$) of the cultures every hour in a kinetic Bio-Tek KC Junior microplate reader (BioTek Instruments, Winooski, USA). The data obtained with the reader were exported to and processed with the Excel-MS software. Cultures used to follow metabolism of *Lact. plantarum* RM71 were performed in CDM by scaling volumes up to 15 ml. The optical densities measured with a microtitre plate reader and with a spectrophotometer were different due to the different optical geometries. The lag phase was defined as the time needed to reach an optical density 10x that of the initial OD$_{600}$. The maximum specific growth rate, $\mu_{\text{max}}$, was determined by calculating the slope of the exponential growth phase ($\mu_{\text{max}} = \Delta \ln(\text{OD}_{600})/\Delta t$). The results were expressed as the means for lag phases, or $\mu_{\text{max}}$, calculated from at least two independent experiments.

**Fermentation in wine-resembling and basal fermentation media**

Fermentations of 15 ml were run at 25ºC in a wine resembling medium (Ugliano, Genovese, & Moio, L. (2003) with the following composition (g l$^{-1}$): glucose (2), fructose (2), tartaric acid (5), acetic acid (0.6), L-malic acid (1.75), yeast extract (2.0), NaCl (0.2), (NH$_4$)$_2$SO$_4$ (1), MnSO$_4$ (0.05), MgSO$_4$.7H$_2$O (0.2), K$_2$PO$_4$ (1) and ethanol (8% v/v). A variant of this medium, named basal fermentation medium (BFM) for convenience, was also used which lacked of tartaric acid, acetic acid and ethanol. Galactose (1 %), instead of glucose and fructose was added as sugar substrate in this medium. Catechin (100 mg l$^{-1}$) was added or omitted as indicated. The initial pH was adjusted at pH 3.8 in either medium and then filter sterilized. Cells grown in MRS medium were washed three times with saline solution (0.85% NaCl) and then inoculated
in wine resembling or BFM media. The bacterial growth was followed daily by plating 100 µl of properly diluted medium on MRS medium (Difco laboratories) adjusted to pH 5.0 before agar addition and sterilization. Plates were incubated aerobically at 25 ºC for three days in a Memmert incubator.

Analysis of catechin degradation by high-performance liquid chromatography-diode array detector (HPLC-DAD)

Potential catechin degradation by Lactobacillus plantarum RM71 was studied in 5 ml of CDM, wine resembling or BFM media cultures. Supernatants for HPLC-DAD analysis were obtained by filtration (pore size 0.22 µm, Millipore) of three mililiters of inoculated medium or from its cell-free control, both after 40 h incubation at 30º C. Catechin was extracted twice from the supernatants with one third of the reaction volume of ethyl acetate (Lab-Scan, Ireland). HPLC-DAD analysis was performed as described previously (Rodríguez et al. 2008). Catechin degradation was followed by comparing retention times and spectral data of the peaks obtained in the chromatograms. Commercial catechin was used as standard.

Fermentation analysis by high-performance liquid chromatography (HPLC)

Supernatants from fermentation experiments were analyzed by HPLC in order to determine the amounts of the sugars galactose, glucose and fructose as well as L-lactic and L-malic acids. A Thermo (Thermo Electron Corp., Waltham, MA) chromatograph equipped with a P-4000 Spectra System Pump, an AS3000 autosampler, a RI-150 refraction index and a UV 6000 LP photodiode array detectors were used. A 1.5 mmol l⁻¹
$\text{H}_2\text{SO}_4$ aqueous solution as mobile phase was applied to an Aminex® HPX-87H column (300 mm x 7.8 mm) (BIO-RAD) at a 0.6 ml/min flow rate at 35 °C. Samples were filtered through a 0.45-µm polyvinylidene difluoride filter, diluted two-fold, and injected in duplicated. Galactose, glucose or fructose detection was performed at 210 nm (refraction index detector) and L-lactic and L-malic acids at 380 nm (diode array detector). Sugar and organic acid standards were used for the identification of the HPLC peaks on the chromatograms, and for the estimation of their concentration by a calibration curve.

RESULTS

Growth behaviour of Lactobacillus plantarum RM71 in control CDM medium

To study the catechin effects on the fermentation performance of *Lact.* plantarum RM71, growth and the time course of sugar consumption were monitored in a chemical defined medium designed for *Lact. plantarum.* With the use of CDM it was ensured that catechin was the only flavonoid in the medium and thus the effects on growth could be more easily ascribed to this flavanol. Furthermore use of CDM would facilitate the evaluation of potential catechin degradation products. As a model growth sugar for CDM experiments we used galactose which is abundant in vegetable fibre and the diet. On this sugar cells grow more slowly than, i.e., glucose and the changes produced by catechin could be more properly monitored.

Before studying catechin effects, we firstly examined the growth behaviour of *Lact. plantarum* RM71 in CDM in absence of catechin. The single effects of citrate and acetate on growth were studied by using four different CDM variants that were prepared
by addition or omission of one or both of these acids in the formulation. The
dependence of growth patterns on pH was relevant to subsequently interpret if catechin
effects were also pH-dependent. Therefore we evaluated this effect by adjusting the
initial medium pH at 5.5 or 6.5 in absence of catechin.

The growth patterns of *Lact. plantarum* RM71 in the four CDM variants at pH
5.5 or 6.5 are shown in Fig. 1. *Lact. plantarum* RM71 grew well in absence of both,
citrate and acetate (panels A and E). The presence of these acids in the medium
prolonged the lag phase (panels B and F) and elicited a biphasic growth pattern that did
only occur in the pH 5.5 CDM (panels B and C). It appeared that citrate was the only
acid leading to this growth profile because the lag phase prolongation (panels B, C, F,
and G) as well as was biphasic growth (panels B and C) were observed only in citrate-
supplemented CDM, regardless of acetate inclusion in the medium.

*Catechin modifies growth and metabolism of Lactobacillus plantarum RM71 to improve
its fermentation performance in CDM*

Results show that the presence of catechin provided advantages to *Lact.*
*plantarum* RM71 for growing in CDM. The two positive effects induced by this
flavanol were shortening of the lag phase and higher final cell densities, both occurring
regardless of the initial pH set-up (Fig.1).

Examination of Fig. 1 revealed that *Lact. plantarum* RM71 achieved higher cell
densities in the catechin-supplemented CDM compared to control. This growth
advantage was more pronounced in the pH 5.5 than in the pH 6.5 CDM. The increase in
cell density reached in the pH 6.5 CDM upon catechin was of less magnitude than at pH
5.5 and it became equal to the cell density of the control soon after entering in the
stationary phase (panels E to H). Contrarily, in cultures grown at initial pH 5.5 the higher cell density reached upon catechin was prolonged for a longer time during the stationary phase (panel A) or maintained steadily higher than the control (panels B to D). The increase in cell density upon catechin was not a dose-dependent event.

Compared to controls growth of *Lact. plantarum* RM71 in catechin-containing medium was quicker upon inoculation in the pH 5.5 CDM (panels A to E) than in the pH 6.5 medium (panels E to F). Thus, cultures carried out, i.e., in complete pH 5.5 CDM supplemented with 100 mg l\(^{-1}\) of catechin, shortened the duration of lag phase by 8 h (± 0.87) compared to control (panel B); while a 4.5 h (± 0.54) shortening was observed at an initial pH 6.5 (panel F). This quicker growth upon inoculation induced by catechin was however not dose-dependent, as the lengths of the lag phases were similar at the two concentrations tested (Fig. 1). We also observed that catechin induced a shift in the growth pattern of *Lact. plantarum* RM71 in complete pH 5.5 CDM. This pattern changed from a biphasic growth displayed in absence of catechin to a diauxic pattern in presence of the flavanol (panel B).

Two key metabolic traits, sugar consumption and lactic acid production, were monitored to evaluate the effect of catechin on *Lact. plantarum* RM71 performance in complete pH 5.5 CDM. The time course of galactose consumption as well as of lactic acid formation, were compared between cultures grown upon 100 mg l\(^{-1}\) of catechin and control cultures lacking the flavanol (Fig. 2). The presence of catechin in CDM gave rise to an increased galactose consumption respect to controls (increase of 39 % when compared to the control lacking catechin). A quicker galactose consumption and lactic acid production were also observed upon catechin. Thus, the galactose consumption rate in the first 48 h was of 5.55 mmol l\(^{-1}\) d\(^{-1}\) for the catechin supplemented cultures in contrast with 3.44 mmol l\(^{-1}\) d\(^{-1}\) of the control. Catechin had little effect on the maximum
rate of galactose consumption as it was, in the period measured and assuming steady-
state conditions, similar in presence (1.05 ± 0.06 mmol l\(^{-1}\) h\(^{-1}\)) or in absence (0.95 ± 
0.05 mmol l\(^{-1}\) h\(^{-1}\)) of the flavanol. Growth was improved by the presence of catechin in 
the medium (Fig. 2) confirming the above results obtained in cultures carried out on 
microtitre plates (Fig. 1) (please note that, as mentioned in the Material and Methods 
section, the optical densities of experiments displayed in Fig. 1 and Fig. 2 are different).

The fermentation performance of Lactobacillus plantarum RM71 in wine-resembling 
medium and basal fermentation medium is improved by catechin

To test the effects of catechin in media relevant to practical fermentation 
processes, firstly a wine-resembling medium set up at an initial pH of 3.8 was 
fermented with Lact. plantarum RM71 strain. This fermentation was monitored for two 
days, time needed for this bacterium to achieve its maximum log increase in this 
medium. Sugar consumption, malic acid degradation and lactic acid production were 
assayed daily in catechin-lacking or catechin-supplemented (100 mg l\(^{-1}\)) media. Lact. 
plantarum RM71 metabolized glucose and fructose simultaneously in both media but 
the total sugar consumption was higher in the medium supplemented with catechin 
compared to control (18.77 vs 21.49 mmol l\(^{-1}\). See Table 1).

The presence of catechin clearly accelerated sugar consumption at the initial 
stage of fermentation (0-24 h) as it could be seen for glucose and fructose (Fig. 3), 
which proceeded about ten and six times faster, respectively, than their controls (Table 
1). The mean consumption rates of glucose and fructose per day during the entire 
fermentation period were of 5.45 mmol l\(^{-1}\) d\(^{-1}\) for glucose (5.29 for fructose) upon 
catechin instead of 4.5 mmol l\(^{-1}\) d\(^{-1}\) for glucose (4.8 for fructose) in absence of the
flavanol. The fermentation of malic acid was faster than that of sugars and completed in only one day. A sample taken at ten hours from the onset of fermentation showed that catechin clearly sped up the consumption of malic acid (Fig. 3). Thus, at this time (10 h), 61.3 % of the total malic acid added (7.15 mmol l⁻¹) was already consumed by Lact. plantarum RM71 in presence of catechin, in contrast with the significantly lower 29.3 % (3.35 mmol l⁻¹) malic acid consumption by the control culture.

Compared to control, the presence of catechin gave rise to an increase of lactic acid production, as it is reflected by the daily and total amounts produced (Table 1). The difference in lactic acid production was specially marked at the onset of fermentation, which could be expected because of the accelerated malic acid and sugar consumption in the catechin supplemented cultures.

To test if catechin exerted similar effects on Lact. plantarum plant-derived fermentations different from wine conditions, a medium named BFM was prepared. The medium was devoid of wine characteristic components, i.e, tartaric acid, ethanol and acetic acid as these were not used by Lact. plantarum RM71 in the fermentation of wine resembling medium (not shown). Furthermore glucose and fructose were replaced by galactose (1%) to investigate if catechin affected the fermentation of other sugars. Galactose is present in the diet and is usually present in dietary fibre from vegetables and fruits, such as the arabinogalactan, which has been shown to increase the Lactobacillus spp. population in the human intestine (Robinson, Feirtag & Slavin (2001)).

As it can be observed in Table 2, the presence of catechin stimulated the growth of Lact. plantarum RM71 in BFM. The patterns of malic acid degradation and galactose utilization are shown in Fig. 4. The fermentation of malic acid was completed in two days regardless catechin supplementation, however the presence of this flavanol clearly
sped up the consumption of this acid. Thus, during the first day of fermentation, 59.8 %
of the total malic acid added was consumed by Lact. plantarum RM71 in presence of
catechin, in contrast with the significantly lower 13.7% by the control culture.

We observed that the extent of sugar utilization by Lact. plantarum RM71 was
increased upon catechin (17.6 vs 29.14 mmol l⁻¹). In addition catechin promoted a faster
galactose consumption respect to controls. The mean consumption rate of galactose per
day during the entire fermentation period was of 9.71 mmol l⁻¹ d⁻¹ upon catechin,
instead of 5.86 mmol l⁻¹ d⁻¹ in absence of the flavanol. Examination of Table 2 revealed
that the presence of catechin promoted earlier galactose utilization. Thus at the initial
stage of fermentation (0-24 h), sugar consumption proceeded at a diminished rate of
0.84 mmol l⁻¹ d⁻¹ in the control cultures, compared to a much faster rate of 4.35 mmol l⁻¹
d⁻¹ upon catechin. Lactic acid production was also monitored as indicator of the
fermentation performance of Lact. plantarum RM71. As it occurred in the wine-
resembling medium, in BFM daily and total lactic acid productions (as well as lactic
acid yields) were higher upon catechin (Table 2), being these increases more
pronounced at the onset of fermentation (0-24h).

Catechin is not metabolized by Lactobacillus plantarum RM71

Spectrometric analysis was carried out to search for potential catechin
degradation/metabolism resulting from the growth of Lact. plantarum RM71.
Supernatants from stationary phase cultures of Lact. plantarum RM71 grown in pH 5.5
CDM, wine- resembling medium or BFM upon 100 mg l⁻¹ of catechin, were compared
with cell-free negative controls incubated under the same conditions. Experiments
performed in CDM appeared in Fig. 5, which displayed practically no differences in the
catechin peak eluting at 18.17 min, among HPLC chromatograms of cell-free control and culture supernatants after 40 h incubation in CDM.

DISCUSSION

The effects of phenolics compounds on lactic acid bacteria have been reported to be rather variable (For a recent review see Rodríguez et al. 2009). This variability depends on the compound (Vivas et al. 1997, Campos et al. 2003, Figueiredo et al. 2008); the strain under study (Figueiredo et al. 2008); or the medium used for growth (Alberto et al. 2001). Up to date some authors have associated the positive effects of food relevant phenolic compounds on growth of lactic acid bacteria, to the degradation of the compound. Thus the growth stimulating effects of gallate on O. oeni (Vivas et al. 1997) or Lact. hilgardii (Alberto et al. 2001) were reported to be dose-dependent and suggested to be linked to gallate degradation. In the case of anthocyanins it was suggested that glucose moiety was used as energy source to justify the positive effect on the growth of O. oeni (Vivas et al. 1997).

In this study it was observed that most of the catechin remained in the media after fermentation with Lact. plantarum RM71. This result was interpreted as this bacterium did not degrade the largest catechin bulk, otherwise the catechin peak would have been disappeared or greatly diminished, and new compounds would have been appeared. Consequently the metabolic and growth stimulations promoted by catechin can not be ascribed to catechin metabolism by Lact. plantarum RM 71. These results are in agreement with those previously reported for O. oeni by Reguant et al. (2000), who also uncoupled the stimulation of growth triggered by catechin from the metabolism of this flavanol. Since O. oeni grows better in absence of oxygen, these
authors suggested that the oxygen scavenging ability of flavonoids tested, including catechin, favoured the growth stimulating effects observed. Contrarily, other authors could not apply the same reasoning as they found that catechin, or epicatechin, showed neutral effects on the growth of *O. oeni* and *Lact. hilgardii* (Figueiredo *et al.* 2008). It has been recently reported that the radical scavenging capacity of catechins increased with the pH of the medium (Muzolf *et al.* 2008), however in this work the positive effects of catechin on the growth of *Lact. plantarum* were more pronounced at the lower pH and not dependent on the dose of catechin used. Therefore the positive influence of catechin on growth found here can hardly be ascribed to its radical scavenging capacity.

By time course of sugar consumption data tracking experiments it is shown that, at least in the case of *Lact. plantarum* RM71, catechin stimulates growth by promoting quicker sugar consumption, increasing the extension of sugar utilization and stimulating malic acid decarboxylation. Clearly this is a different mode of action of catechin to that suggested by this flavanol on *Lact. hilgardii* where the growth stimulating effects were linked to catechin degradation (Alberto *et al.* 2001, Figueiredo *et al.* 2008); or to that suggested by Reguant *et al.* (2000) for *O. oeni* who justified the stimulating effects on the antioxidant properties of catechin.

The beneficial effects on growth were observed regardless of the catechin concentration examined, which support again the hypotheses that growth stimulation is uncoupled from catechin degradation. The lack of dose dependent effects is in agreement with the weak bactericidal activity (Nakayama *et al.* 1998) resulting from the shallow location, close to the phospholipid/water interface, previously reported for catechin (Caturla *et al.* 2003). Otherwise an increased bactericidal effect would be expected at increased catechin concentrations, as it occurs with galloylated catechins which possess a higher insertion capacity in the membrane (Ikagai *et al.* 1993, Caturla...
Although the growth advantages were observed regardless the initial pH values, they were more pronounced in the pH 5.5 CDM which could indicate a stronger interaction of catechin with membrane phospholipids at this lower pH than at pH 6.5.

According to previous reports (Kajiya et al. 2001, Caturla et al. 2003) catechin does not enter the cell, so it is probable that the effects observed on growth and metabolism were caused by alteration of the biophysical properties of the membrane of Lact. plantarum RM71. It is possible that catechin, after had caused its biological effect, could had left the membrane by passive diffusion along time, a process that has been previously described for other flavonoids such as quercetin (Movileanu et al. 2000).

Thus catechin, once located in the membrane, could alter the function of proteins associated with the bilayer and affect the transport of materials across it. Sugar and malate transporters seem to be affected proteins, accordingly to the changes in sugar and malic acid consumption observed here upon catechin. The citrate transport system could be affected as well, since catechin triggered variations in the growth patterns displayed by Lact. plantarum RM71 on citrate supplemented CDM. Experiments are currently under way to determine if catechin alters some biophysical properties of the membrane of Lact. plantarum and affects these transport systems.

Citrate prolonged lag phase and induced sequential growth behaviour in CDM. The scope of this study was not to investigate this behaviour, however the long lag phase observed upon citrate indicates that there might be some limitations on the substrate utilization apparatus at the onset of cultures, which is usual when growing in mixed-substrate environments (Daae et al. 1998). The biphasic growth could be due to a sequential metabolism of carbon sources since this behaviour was only observed at an initial pH of 5.5, which is close to the optimum pH range for citrate metabolism in Lact. plantarum (Palles et al. 1998). This type of sequential metabolism is not unprecedented.
for *Lactobacillus* ssp. Thus, diauxic growth has been described during growth, also under sugar limitation, on a mixture of sugars and fructooligosaccharides (FOS) (Goh *et al.* 2007) and glucose and citrate (de Figueroa *et al.* 1996). Catechin shortened the lag phase so it is probable that this flavanol relieved limitations in substrate utilization. Moreover in complete pH 5.5 CDM, catechin replaced the biphasic growth by a diauxic growth where a clear diauxic lag was evident. This growth pattern is usually associated to a better control of substrate assimilation (Daee *et al.* 1998) so if catechin, as it is suspected, inferred a more efficient substrate uptake, a diauxic lag would be necessary to fit the enzyme synthesis for the utilization of the second substrate.

The results obtained in wine resembling medium or BFM revealed that catechin sped-up malolactic fermentation, stimulated the consumption of several sugars as well as the lactic acid formation, thus providing growth advantages to *Lact. plantarum* RM71 in these media. Growth stimulation linked to malolactic fermentation is known to be caused by its coupling to ATP synthesis by either, the entry through F(H+)-ATPase of environmental protons generated in the malate decarboxilation process; and the malate/lactate antiport coupled also to F(H+)-ATPase. Accordingly, the growth improvement of *Lact. plantarum* RM71 upon catechin should not be surprising in view of the accelerated malolactic fermentation and lactic acid production provided by the flavanol. Similar effects have been also described for the growth of *O. oeni* CECT 4101, where this flavanol was found to stimulate the malolactic fermentation development (Reguant *et al.* 2000). Although malolactic fermentation is classically associated to winemaking, the importance of the growth benefit obtained by *Lact. plantarum* from the utilization of malic acid should not be restricted to the wine environment but extended to other fermented food plants, as recently reported (Plumed-Ferrer *et al.* 2008). These authors found a significantly higher growth of *Lact.*
plantarum in cucumber juice compared to the rich MRS medium and highlighted the crucial importance of the higher decarboxylation of malic acid in this stimulation.

In summary, the results presented here show that catechin improved malic acid degradation, fermentation of several sugars and therefore *Lact. plantarum* RM71 growth, which are desirable traits for a successful performance of this species as starter in several food plant fermentations. These stimulations are not linked to catechin degradation.

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Legends to Figures.

**Fig. 1.** Catechin effects on the growth curves of *Lactobacillus plantarum* RM71 depend on the initial pH and CDM formulation. Selected growth curves of cells grown at 30°C in pH 5.5 (panels A to D) or in pH 6.5 (panels E to H) CDM, are shown. Panels A to D or E to H, correspond consecutively to growth in CDM lacking both, citrate and acetate (A, E), including citrate and acetate (B, F), including citrate only (C, G) and acetate only (D, H). Catechin was added at the following concentrations: △, no catechin (controls); ◆, 100 mg l^{-1}; ■, 200 mg l^{-1}.

**Fig. 2.** Time course of galactose consumption and lactic acid production by *Lactobacillus plantarum* RM71 in the presence of catechin. Cultures (15 ml) of *Lact. plantarum* RM71 were grown in pH 5.5 CDM supplemented with 0.5% galactose. Growth was carried out in presence (■) or in the absence (□) of 100 mg l^{-1} of catechin. Triangles represent the time course of galactose consumption in the presence (▲) or absence (△) of catechin. Diamonds represent the time course of lactic acid production in the presence (◆) or absence (◇) of catechin. Values are the average of three independent replicates with maximum standard deviation of <7%.

**Fig. 3.** Catechin accelerates sugar consumption, malolactic fermentation and lactic acid production during growth of *Lactobacillus plantarum* RM71 in wine resembling medium. The wine resembling medium was set-up at an initial pH of 3.8 and fermented with *Lact. plantarum* RM71 for two days at 25°C. Monitoring of the fermentation metabolic parameters was carried out in cultures grown in presence of catechin (100 mg l^{-1}) (solid symbols) or in its absence (open symbols). Symbols: Fructose (▲, △);
Glucose (■,□); L-lactic acid (◆,◇); L-malic acid (●,○). Each point is the average value of at least two independent determinations. Standard deviations are represented by vertical bars.

Fig. 4. Effects of catechin on fermentation metabolic traits of *Lactobacillus plantarum* RM71 in basal fermentation medium (BFM). The medium was set-up at an initial pH of 3.8 and fermented with *Lact.plantarum* RM71 for three days at 25°C. Monitoring of the fermentation metabolic parameters was carried out in presence of catechin (100 mg l\(^{-1}\)) (solid symbols) or in its absence (open symbols). Triangles represent L-malic acid (▲,△); squares, galactose (■,□); diamonds, L-lactic acid (◆,◇). Each point is the average value of at least two independent determinations. Standard deviations are represented by vertical bars.

Fig. 5. HPLC chromatograms of *Lactobacillus plantarum* strain RM71 supernatants grown in the presence of catechin. Supernatants of *Lact. plantarum* RM71 cultures and cell-free control culture after 40 h incubation in CDM, were analyzed by HPLC as described. The medium was set-up at an initial pH of 5.5 and 100 mg l\(^{-1}\) of catechin in absence (A), or in presence of cells (B). Absorbance was recorded at 280 nm.
Table 1. The fermentation performance of *Lactobacillus plantarum* RM71 in wine-resembling medium is improved by catechin.

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>Glucose mmol l⁻¹</th>
<th>Fructose mmol l⁻¹</th>
<th>L-malic mmol l⁻¹</th>
<th>L-lactic mmol l⁻¹</th>
<th>Log c.f.u</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>0.2 (2.2) *</td>
<td>0.38 (4) †</td>
<td>11.65</td>
<td>9.7 (1.08) ‡</td>
<td>4.62 §</td>
</tr>
<tr>
<td>1-2</td>
<td>8.9 (97.8)</td>
<td>9.29 (96)</td>
<td>0.0</td>
<td>25.23 (0.7)</td>
<td>7.3</td>
</tr>
<tr>
<td>Total</td>
<td>9.1</td>
<td>9.67</td>
<td>11.5</td>
<td>34.93 (0.78)</td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>2.22 (20.4)</td>
<td>2.25 (21.2)</td>
<td>11.41</td>
<td>15.55 (0.9)</td>
<td>5.05</td>
</tr>
<tr>
<td>1-2</td>
<td>8.68 (79.6)</td>
<td>8.34 (78.7)</td>
<td>0.0</td>
<td>28.45 (0.9)</td>
<td>7.7</td>
</tr>
<tr>
<td>Total</td>
<td>10.9</td>
<td>10.59</td>
<td>11.78</td>
<td>44 (0.86)</td>
<td></td>
</tr>
</tbody>
</table>

Consumption or production values are calculated using the average values represented in Fig. 3. *, † Values in parentheses are percentages of glucose, fructose or L-malic acid consumed respect to total amount used by *Lact. plantarum* RM71 in the fermentations. ‡ Values in parentheses represent the daily or total lactic yield by glucose, fructose and L-malic acid consumed (theoretical value of the ratio lactic/malic acid =0.67). CAT (cultures grown upon 100 mg l⁻¹ of catechin). § Consumption or production values and c.f.u. counts are the average of at least two independent fermentations. Values do not differ significantly by using non parametric tests (*P*<0.05).
Table 2. The fermentation performance of *Lactobacillus plantarum* RM71 in basal fermentation medium (BFM) is improved by catechin.

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>Galactose consumption</th>
<th>L-malic consumption</th>
<th>L-lactic production</th>
<th>Log c.f.u</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>0.84 (4.8)*</td>
<td>1.59 (13.7)†</td>
<td>0.78 (0.28)‡</td>
<td>3.87 §</td>
</tr>
<tr>
<td>1-2</td>
<td>10.32 (58.6)</td>
<td>9.99 (86.3)</td>
<td>15.07 (0.55)</td>
<td>6.04</td>
</tr>
<tr>
<td>2-3</td>
<td>6.44 (36.6)</td>
<td>0 (0)</td>
<td>7.61 (0.59)</td>
<td>8.48</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>17.6</td>
<td>11.58</td>
<td>22.68 (0.53)</td>
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</tr>
<tr>
<td><strong>CAT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>4.35 (14.9)</td>
<td>6.83 (59.8)</td>
<td>5.57 (0.42)</td>
<td>4.27</td>
</tr>
<tr>
<td>1-2</td>
<td>14.2 (48.7)</td>
<td>4.59 (40.2)</td>
<td>26.37 (0.84)</td>
<td>6.7</td>
</tr>
<tr>
<td>2-3</td>
<td>10.59 (36.3)</td>
<td>0 (0)</td>
<td>12.3 (0.58)</td>
<td>8.76</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>29.14</td>
<td>11.42</td>
<td>38.66 (0.58)</td>
<td></td>
</tr>
</tbody>
</table>

Consumption or production values are calculated using the average values represented in Fig. 4. * Values in parentheses are percentages of galactose or L-malic acid consumed respect to total amount used by *Lact. plantarum* RM71 in the fermentations. † Values in parentheses represent the daily or total lactic yield by galactose and L-malic acid consumed (theoretical value of the ratio lactic/malic acid =0.67). CAT (cultures grown upon 100 mg l⁻¹ of catechin). § Consumption or production values and c.f.u. counts are the average of at least two independent fermentations. Values do not differ significantly by using non parametric tests (*P*<0.05).
Fig. 1

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Fig. 2

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![Graph showing growth (OD600) and galactose and L-lactic acid concentrations over time.](image-url)
Fig. 3

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![Graph showing the relationship between time (days) and concentrations of glucose, fructose, L-lactic acid, and L-malic acid.](image-url)
Fig. 4

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