Optimization of chemically defined medium for the study of anaerobic and respiratory growth in *Lactobacillus plantarum* and *L. casei*

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INTRODUCTION

Studies on the growth adaptation of *Lactobacillus plantarum* (Stevens et al. 2008, Tseusink et al. 2009, Bron et al. 2012) revealed that aerobic (air) and respiratory (air, heme and menaquinone) cultivations significantly modifies the gene expression and metabolic pathways, resulting in phenotypes with altered stress response and technological properties. Recently, Zotta et al. (2014) investigated aerobic and respiratory growth in the *L. casei* group.

L. plantarum and *L. casei*, being able to synthesize most of amino acids (except the branched-chain amino acids), are well adapted to different ecological niches and growth conditions.

However, we verified that the chemically defined medium (CDM) used in several studies (Teusink et al. 2005; Wegkamp et al. 2010) on *L. plantarum* poorly supported aerobic and respiratory growth of *L. plantarum* and *L. casei,* suggesting that different nutritional requirements are needed for anaerobic and respiratory cells.

AIM of work: optimization of CDM composition for comparative studies of anaerobic and respiratory metabolism in potential respiring lactobacilli.

MATERIALS AND METHODS

Growth conditions: Growth was carried out in anaerobic (**AN**) and respiratory (air, supplementation with 2.5 µg/mL hemin and 1 µg/mL menaquinone; **RS**) conditions, using modifications of the chemically defined medium (CDM) used in several *L. plantarum* studies (Teusink et al. 2005). Any changes to the original CDM (CDM1) have been reported in the different experimental STEPS (see below).
Growth in STEP 1 to 3 and STEP 5 was performed in 24-well microplates (static or shaken, respectively for AN and RS condition), those in STEP 4 in static tubes (AN) or shaken baffled flasks (RS).

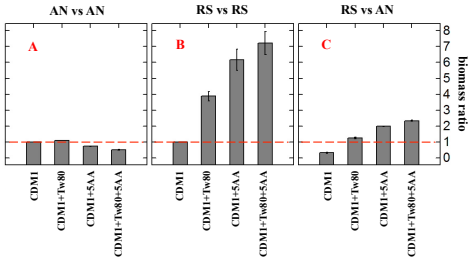
Factorial design: The effect of Tween 80, L-alanine, L-asparagine, L-aspartate, L-proline and L-serine (missing in CDM1) on the growth performances of *L. casei* N87 was investigated with a 2^5 factorial experiment (32 run x 2 replicates) in AN and RS conditions.

Changes in membrane fatty acids and volatile compounds: composition of membrane fatty acids (degree of unsaturation and length of FA chains) in AN and RS cells, grown with or without Tween 80, was analyzed by Gas-Chromatography (GC), while volatile compounds were measured by Solid Phase Micro Extraction (SPME) GC-MS technique.

Adequacy of modified CDM in the growth of *L. plantarum* and *L. casei* groups: Twenty-one strains belonging to *L. plantarum* group (7 *L. plantarum*, 1 *L. plantarum* subsp. *argentoratensis*, 2 *L. paraplantaru*,; 1 *L. pentosus*; Guidone et al. 2013) and *L. casei* group (4 *L. casei*, 3 *L. rhamnosus*, 3 *L. paracasei*; Zotta et al. 2014) were grown in CDM1, with or without Tween 80 and/or Asn+Pro+Ser, under AN and RS conditions, to verify the effectiveness of optimized CDM.

Effect of Tween 80 and L-alanine, L-asparagine, L-aspartate, L-proline and L-serine on the anaerobic and respiratory growth of L. casei N87

STEP 1: We assumed that Tween 80 (70% oleic acid) and L-alanine (Ala), L-asparagine (Asn), L-aspartate (Asp), L-proline (Pro) and L-serine (Ser), the missing nutrients in CDM (Teusink et al. 2005, following indicated as **CDM1**), might limiting for growth of *L. plantarum* and *L. casei* under respiratory conditions (air and supplementation with heme and menaquinone).
Tween 80 and the pool of 5 aminoacids (Ala+Asn+Asp+Pro+Ser) were added to CDM1 and the anaerobic (**AN**) and respiratory (**RS**) growth of *L. casei* N87 was evaluated.

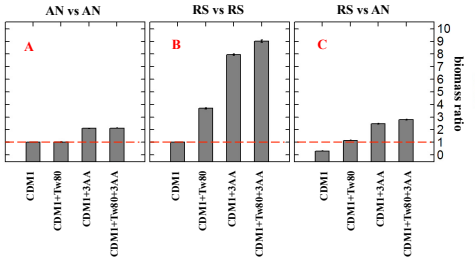


Ratio between production of biomass (g/L) in CDM1 supplemeted with Tween 80 and/or amino acids vs CDM1. Cultivation in CDM1-AN was used as reference in plots A and C; cultivation in CDM1-RS was used as reference in plot B.

• Supplementation with Tween 80 did not affect growth under AN condition, while the presence of 5 amino acids slightly reduced growth in AN cultures (plot A)
• On the contrary, Tween 80 and amino acids increased the biomass of RS cultures, respectively, 4 times (from 0. 2 g/L in CDM1 to 0.8 g/L in CDM1+Tw80) and 6 times (from 0.2 g/L in CDM1 to 1.2 g/L in CDM1+5AA). Combination of Tween80 and 5AA provided a further gain (7 times) in growth performances (plot B)
• As expected, un-supplemented CDM1 supported to a limited extend (< 3 times compared to AN cultivation) the respiratory growth, while supplementation with Tween80 and/or 5 AA enhanced biomass production (plot C)

STEP 2: Since the supplementation with 5 amino acids increased the RS growth but slightly impaired the AN one, the effect of Ala, Asn, Asp, Pro and Ser (singularly or in combination) was evaluated in AN and RS cultivations in a factorial design.
The results demonstrated that Asn+Pro+Ser (related to TCA cycle and pyruvate precursor pathways) was the best amino acid pool to stimulate both AN and RS cells. Supplementation only with Ala, Asn and Asp (singularly or in combination) even decreased AN and RS growth compared with CDM1. Asn provided advantage in biomass production when combined with Ser and/or Pro.

STEP 3: The effect of Asn+Pro+Ser (3AA,) in combination or not with Tween 80, was evaluated in AN and RS conditions.

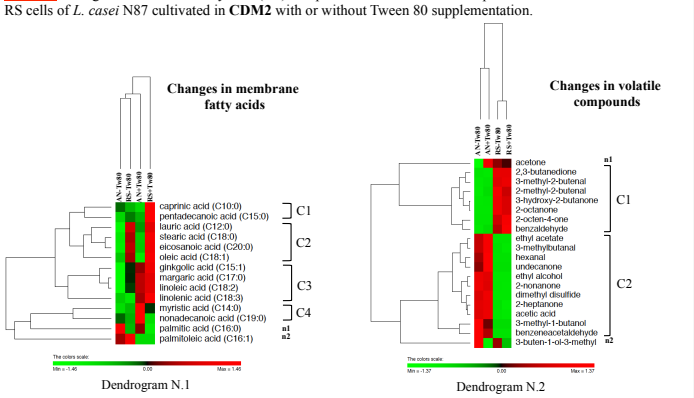


Ratio between production of biomass (g/L) in CDM1 supplemeted with Tween 80 and/or amino acids vs CDM1. Cultivation in CDM1-AN was used as reference in plots A and C; cultivation in CDM1-RS was used as reference in plot B.

• As shown in **Step 1**, Tween 80 did not affect AN growth, but supplementation with 3AA doubled the biomass production in AN cultivation (plot A)
• The trend of growth in RS condition was similar to that reported in **Step 1**, even if the addition of 3AA slightly increased the growth compared with supplementation of 5AA pool (7 times, from 0.2 g/L in CDM1 to 1.4 g/L in CDM1+3AA; 9 times, from 0.2 g/L in CDM1 to 1.8 g/L in CDM1+Tw80+3AA) (plot B)
• Since biomass production was similar (1.4 g/L) in both AN and RS conditions with 3AA, we assumed that Tween 80 was the factor that differently affected the AN (no effect) and RS (stimulating effect) growth.
• CDM1 supplemented with 3AA (**CDM2)** was further used for further experiments.

Effect of Tween 80 on the composition of membrane fatty acids and volatile compounds in anaerobic and respiratory cultures of L. casei N87

STEP 4: Changes in membrane fatty acid (FA) composition and in volatile compounds were evaluated in AN and RS cells of *L. casei* N87 cultivated in **CDM2** with or without Tween 80 supplementation.



Matrix Hierarchical Cluster Analysis was performed with PermutMatrix program v.1.9.3 (LIRMM, France). Normalization of variable dataset, Euclidean distance and McQuitty's linkage (WPGMA) method were used to perform clustering. Column dendrogram: AN-Tw80, AN growth without Tween 80 supplementation; RS-Tw80, RS growth without Tween 80 supplementation; AN+Tw80, AN growth with Tween 80 supplementation; RS+Tw80, RS growth with Tween 80 supplementation. Row dendrogram N1: fatty acids; row dendrogram N2: volatile compounds. Colour scale: from green (negative data; minimum value is -1.46 and -1.37, respectively, for dendrogram N.1 and dendrogram N.2) to red (positive data, maximum values is +1.46 and +1.37, respectively, for dendrogram N.1 and dendrogram N.2), indicates the change from the mean in standard deviation units.

Fatty acids

• Respiration increased the degree of desaturation of FAs
• The presence of Tween 80 enhanced the percentage of polyunsatured FAs (linoleic and linolenic acids) in both AN and RS conditions and of monounsatured FAs (ginkgolic, oleic) only in RS cells
• Monounsatured palmitoleic acid dramatically decreased in AN and RS growth supplemented of with Tween 80

Volatile compounds

• Tween 80 did not affect the production of volatile compounds (with exception of acetone and 3-buten-1-ol-3-methyl), which was mainly correlated to the growth condition
• 2,3-butanedione (diacetyl), 2-methyl-2-butenal, 3-methyl-2-butenal and benzaldehyde were principally synthesised by RS cells, while 2-nonanone, 2-heptanone and acetic acid were the most prominent compounds of AN cultures

STEP 5: ***Effect of Tween 80 and* Asn+Pro+Ser *on the anaerobic and respiratory growth of strains belonging to the L. casei and L. plantarum groups***

Supplementation of CDM1 with Tween 80 and Asn+Pro+Ser increased the respiratory growth in all strains, with the exception of the 2 *L. paraplantarum* (unable to growth in CDM1, with or without supplementations, in RS conditions) and the 3 *L. paracasei* (unable to growth in CDM, with or without supplementations, in AN and RS conditions).

CONCLUSIONS

➢ L-alanine, L-proline and L-serine are stimulatory for anaerobic and respiratory growth.
➢ Tween 80 did not affect anaerobic cultivation but is stimulatory for the growth in presence of oxygen, heme and menaquinone (when a possible electron transport chain may be activated). The increase of desaturation that occurs in RS cultures, being correlated to the membrane fluidity, may positively affect the proton and cation permeability.
➢ Respiratory cultures may be exploited for flavour development in fermented foods (i.e. production of diacetyl in dairy products).
➢ The optimised CDM1 (CDM2+Tween 80) may be used for the studies on the metabolism of respiring lactobacilli.

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