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Abstract

Nutritional dependencies, especially those regarding nitrogen sources, govern numerous microbial positive interactions. As for lactic acid bacteria (LAB), responsible for the sanitary, organoleptic, and health properties of most fermented products, such positive interactions have previously been studied between yogurt bacteria. However, they have never been exploited to create artificial co-cultures of LAB that would not necessarily coexist naturally, *i.e* from different origins. The objective of this study was to promote LAB positive interactions, based on nitrogen dependencies in co-cultures, and to investigate how these interactions affect some functional outputs, *e.g.* acidification rates, carbohydrate consumption, and volatile compound production. The strategy was to exploit both proteolytic activities and amino acid auxotrophies of LAB. A chemically defined medium was thus developed to specifically allow the growth of six strains used, three proteolytic and three non-proteolytic. Each of the proteolytic strains, *Enterococcus faecalis* CIRM-BIA2412, *Lactococcus lactis* NCDO2125, and CIRM-BIA244, was co-cultured with each one of the non-proteolytic LAB strains: *L. lactis* NCDO2111, *Lactiplantibacillus plantarum* CIRM-BIA465 and CIRM-BIA1524. Bacterial growth was monitored using compartmented chambers to compare growth in mono- and co-cultures. Acidification, carbohydrate consumption and volatile

compound production was evaluated in direct co-cultures. Each proteolytic strain induced different types of interactions: either strongly positive, weakly positive, or no interactions, with *E. faecalis* CIRM-BIA2412, *L. lactis* NCDO2125 and *L. lactis* CIRM-BIA244, respectively. Strong interactions were associated with higher concentrations in tryptophan, valine, phenylalanine, leucine, isoleucine, and peptides. They led to faster acidification rates, lower pH, higher raffinose utilization and concentrations in five volatile compounds.

Importance:

Lactic acid bacteria (LAB) interactions are often studied in association with yeasts or propionibacteria in various fermented food products and the mechanisms underlying their interactions are being quite well characterized. Concerning interactions between LAB, they have mainly been investigated to test antagonistic interactions. Understanding how they can positively interact could be useful in multiple food-related fields: production of fermented food products with enhanced functional properties or fermentation of new food matrices. This study investigates the exploitation of the proteolytic activity of LAB strains to promote positive interactions between proteolytic and non-proteolytic strains. The results suggest that proteolytic LAB do not equally stimulate non-proteolytic LAB and that the stronger the interactions between LAB are, the more functional outputs we can expect. Thus, this study gives insight into how to create new associations of LAB strains and to guaranty their positive interactions.

Keywords: lactic acid bacteria, positive interactions, commensalism, nutritional dependency, nitrogen nutrition, functional outputs

1 Introduction

Lactic acid bacteria (LAB) are the most prevalent bacterial actors in fermented foods consumed in Western countries (1). LAB can produce a variety of compounds including weak organic acids, e.g. lactic and acetic acids, aroma compounds such as diacetyl, amino acids, peptides, exopolysaccharides, vitamins, as well as hydrolytic enzymes, hydrogen peroxide and bacteriocins, during fermentations (2). These compounds provide fermented foods with varied desirable functional outputs such as organoleptic, sanitary, nutritional, probiotic, and health properties (3–6). However, their production in LAB-fermented foods is both species- and strain-dependant and, consequently, the "super strain" that would produce all the expected metabolites does not exist (7). To increase the functional outputs, we need to find efficient ways to associate strains with complementary properties in an artificial co-culture, defined as an association of microorganisms that may not necessarily be found in nature (8). Co-culture can also increase substrate conversion, yields and microbial fitness, in particular when microorganisms interact positively with each other, either through commensalism, cooperation, mutualism, or syntrophy (8).

The exploitation of nutritional dependencies seems to be one the most promising way for LAB to interact in co-culture. The exchange of nitrogen compounds is of particular interest either as public goods, defined as the pool of molecules available in the medium (9), or through cross-feeding, *i.e* the phenomenon by which one microorganism takes in a primary substrate and converts it into a product excreted as a public good (10). For example, dependencies based on nitrogen nutrients have been observed between the yeast *Saccharomyces cerevisiae* and the LAB *Lactococcus lactis* (11), as well as between the two LAB species, used as a prime example, *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, associated in yogurt (12). In milk fermentation, the sharing of extracellular protease activity, especially by LAB species such as *L. lactis*, is

paramount to ensure microbial interactions in cheese and fermented milks (13). Protease activity is unevenly distributed among the strains, rendering the growth of the strains that lack this activity dependent on the composition of the medium in peptides and free amino acids.

LAB co-cultures were recently shown to efficiently ferment mixes of milk and plant-based substrates (14, 15) to meet the growing need of new substrate use in the context of food transition. Up to now, most LAB strains were mainly selected from dairy applications, but we need to find more suitable candidates to ferment plant-sourced substrates as well. Efficient co-cultures of LAB strains adapted to both dairy and plant-based resources has been achieved by our team in a previous work in which functional complementarity of selected LAB strains were exploited to design co-cultures able to ferment mixes of milk and lupin (15). More specifically, we associated non-proteolytic strains able to degrade specific carbohydrates with proteolytic strains, the latter being expected to provide the former with nitrogen nutrients. As the culture medium was initially rich in nitrogen compounds, and four to six strains were associated in co-culture, this complicated the study of the nitrogen-related interactions between the LAB.

The objective of this study was to promote LAB positive interactions based on nitrogen dependencies in artificial co-cultures and to investigate the possible functional outputs of such interactions. For this, LAB strains were deliberately associated in a model medium mimicking a mix of milk and plant-based substrate. We selected two types of strains from various dairy and non-dairy/vegetable origins. The first ones, referred as donor strains, were selected for their proteolytic activity and their contribution to the development of flavor. *Lactococcus lactis* and *Enterococcus faecalis* were found to be adequate candidates. Both species possess a very efficient proteolytic system and can modify sensorial profiles in cheeses and other fermented dairy products (16, 17). *E. faecalis* proteolytic system was even shown to be

responsible for a decrease of allergenicity of bovine milk proteins (18). The second type of strains, referred to as receiver strains, were selected, in contrast, for their lack of proteolytic activity and their ability to consume raffinose-family oligosaccharides, which are responsible for intestinal discomfort. Strains of *L. lactis* and *Lactiplantibacillus plantarum* that were previously shown to exhibit this capacity (15) were selected for the present study (**Figure 1**).

In the present study, we first developed a chemically defined medium that contained all necessary vitamins, minerals, nucleic acids, as well as the proteins and carbohydrates of bovine milk and lupin. Second, we associated a donor strain with a receiver one. We used either compartmented chambers or direct co-cultures to study LAB interactions and compared them to monocultures. The present study demonstrated that it is possible to promote positive interactions based on nutritional dependencies between LAB strains by exploiting both their proteolytic activities and their amino acid auxotrophies. Regarding the functional outputs that result from LAB interactions, we investigated in particular the acidification rates to ensure safety, RFO consumption to reduce intestinal discomfort, and production of aroma compounds to improve sensory properties. The results indicate that different proteolytic LAB do not equally stimulate non-proteolytic LAB. They further suggest that the stronger the interactions between LAB are, the more functional outputs we can expect.

2 Results

2.1 Selection of the proteolytic (donor) and non-proteolytic (receiver) strains and validation of a chemically defined medium to study LAB interactions

The donor strains were selected according to their proteolytic profiles, *i.e* the NH_2-containing compounds they provided, when grown in the chemically defined medium that contained casein and/or lupin proteins as sole nitrogen source (CDM PROT). The concentrations of free amino acids (FAA) and peptides in the medium after fermentation by each of the donor

strains are illustrated in **Figure 2**. FAA and peptides resulted from the hydrolysis of casein and/or lupin proteins present in the CDM PROT. *E. faecalis* CIRM-BIA2412 (Efa2412), appeared as the most proteolytic strain, with proteolytic indices (PI) of 10.8 % ± 0.1 versus 5.5 % ± 0.1 and 3.6 % ± 0.3 for *L. lactis* NCDO2125 (Lla2125) and *L. lactis* CIRM-BIA244 (Lla244), respectively.

The concentration of total FAA in CDM PROT increased from 4.8 mg/L initially to 250, 261, and 155 mg/L after fermentation by Efa2412, Lla2125 and Lla244, respectively. The concentration of peptides released by Efa2412 (48.8 mg NH_2/L) was significantly higher, compared to that released by the two other donor strains Lla244 (5.8 mg NH_2/L) and Lla2125 (4.0 mg NH_2/L). Among all the free NH_2 groups available in medium after the fermentation by Efa2412, Lla2125 and Lla244, peptides represented 59.4%, 9.6% and 20.7%, for each donor strain respectively.

Figure 3 shows that the three donor strains, Efa2412, Lla2125 and Lla244 in monocultures (dotted lines, lower panel) grew well in the CDM PROT medium, reaching a population of 9.1 ± 0.2, 9.0 ± 0.1 and 9.3 ± 0.1 log(CFU/mL), respectively, after 24 h of culture. In contrast, the three strains *L. lactis* NCDO2111 (Lla450), *L. plantarum* CIRM-BIA465 (Lpl465) and *L. plantarum* CIRM-BIA1524 (Lpl1524) did not grow in CDM PROT in monocultures (dotted lines, upper panel). They maintained their cultivable population at the initial level of inoculation after 24 h of culture, and the pH of the medium remained steady.

To validate that the factor limiting the growth of the three non-growing strains was the nitrogen source, we added 0.5 g/L of casein tryptone, which supplies casein peptides and free amino acids, in the CDM PROT medium, and referred as CDM TRYP. Growth of the strains Lla450, Lpl465 and Lpl1524 was then restored after 24 h as their cultivable population

reached 9.1 ± 0.1, 8.8 ± 0.2, and 8.9 ± 0.1 log(CFU/mL), respectively. These three strains were then considered as non-proteolytic and referred to as receiver strains.

The CDM TRYP, in which all receiver strains grew, contained 108.9 mg/L of FAA and a peptide concentration estimated at 7.7 mg NH_2/L (**Figure 2**). It was therefore considered as a positive control for supporting receiver strain growth. The total nitrogen content of CDM TRYP was lower compared to that of the three donor strains. Thus, at first glance, all three donor strains would be capable of providing the receiver strains enough nitrogen compounds for their growth.

2.2 Bacterial growth in co-cultures

The maximal counts of the three donor strains Efa2412, Lla2125 and Lla244 were not impacted by the mode of culture, neither mono nor co-culture (**Figure 3**). They reached in 14 h a plateau of 9.0 ± 0.2 log(CFU/mL) in 8 h, 9.0 ± 0.2 log(CFU/mL) in 12 h, and 9.2 ± 0.1 log(CFU/mL), respectively. However, the population of Lla2125 decreased significantly after reaching the plateau in co-culture with Lla450 and Lpl465.

In co-culture with Efa2412, the counts of Lla450, Lpl465 and Lpl1524 reached a maximum of 9.0 ± 0.1 log(CFU/mL) within 10 h, 8.9 ± 0.3 log(CFU/mL) in 22 h and 9.0 ± 0.1 log(CFU/mL) 24 h culture, respectively (**Figure 3**). In co-culture with Lla2125, the counts of Lla450, Lpl465 and Lpl1524 reached a maximum of 8.8 ± 0.2 log(CFU/mL) in 20 h, 8.5 ± 0.6 log(CFU/mL) in 22 h and 8.4 ± 0.2 log(CFU/mL) in 22 h, respectively. The three receivers grew at a higher rate in co-culture with the donor strain Efa2412 compared to the donor strain Lla2125. In co-culture with Lla244, the counts of Lpl1524 remained at the inoculation level of 7.0 ± 0.4 log(CFU/mL), whereas the counts of Lla450 and Lpl465 decreased to 6.5 ± 0.1 log(CFU/mL) (**Figure 3**).

To summarize, three types of interactions were observed between the receiver strains and the donor strains, according to the donor strain: strong interactions with Efa2412, weak interactions with Lla2125, and no interaction with Lla244.

We checked that bacterial growth was equivalent in co-cultures performed in compartmented chambers and in direct co-cultures. We chose the specific incubation time of 14 h for this verification because it corresponded to the end of exponential growth phase of the donor strains and the middle of the exponential growth phase of the *L. plantarum* receiver strains. This receiver species was chosen to facilitate count in direct culture on both different plate media for donor and receiver. No significant differences were observed between the bacterial counts obtained in direct co-cultures and in compartmented chambers. Therefore, we chose to use direct co-cultures to study the impact of co-cultivation on acidification, carbohydrates hydrolysis, and volatile profiles, for the rest of the study.

2.3 Acidification parameters were influenced by the type of the interactions and the intrinsic capacities of the receiver strains

Table 1. Maximal acidification rates (dpH/h expressed as absolute values), and pH of CDM PROT after 24 h culture of LAB strains.

Receiver strain	*Donor strain*			
	No	**Efa2412**	**Lla2125**	**Lla244**
	Maximal acidification rates (dpH/h)			
No	-	0.60^{cd}	1.14^{a}	1.05^{ab}
Lla450	0.53^{d}	0.99^{b}	0.97^{b}	1.04^{ab}
Lpl465	0.35^{e}	0.69^{cd}	1.11^{ab}	1.01^{ab}
Lpl1524	0.21^{e}	0.69^{c}	1.09^{ab}	1.02^{ab}
	Final pH after 24 h			
No	-	4.20^{gh}	4.12^{f}	3.94^{ab}
Lla450	4.16^{g}	4.07^{de}	4.10^{ef}	3.94^{ab}
Lpl465	4.22^{h}	3.96^{b}	4.04^{cd}	3.93^{ab}

Lpl1524	4.55^{i}	4.01^{c}	4.08^{def}	3.91^{a}

Donor strains Lla244, Lla2125 and Efa2412 were incubated as monocultures and in co-cultures with Lla450, Lpl465 or Lpl1524. Results for the LAB receiver strains Lla450, Lpl465, Lpl1524 in monoculture were obtained in the CDM TRYP, which corresponds to CDM PROT supplemented with 0.5 g/L of casein tryptone. For strain codes see Table 4. Values followed by different letters indicate significant differences between cultures ($p < 0.05$).

The acidification rates of the different LAB strains in mono- and co-cultures are shown in **Table 1** as well as the final pH observed after 24 h of fermentation. In monoculture in CDM PROT, the donor strains Lla2125 and Lla244 showed the fastest acidification, compared to Efa2412. Among the receiver strains, Lla450 showed a faster acidification than Lpl465 and Lpl1524.

The acidification rates of the co-cultures depended on the receiver strains associated with the donor strains. The two *L. plantarum* strains did not impact the acidification rates of the donor strains with which they were co-cultured. On the contrary, different acidification rates were observed for Lla450 depending on the associated donor strains. Lla450 induced a +65% increase of the maximal acidification rate in co-culture with Efa2412, while a 15% decrease was observed with Lla2125 and no change of the acidification rate in co-culture with Lla244. The final pH in monocultures ranged from 3.94 to 4.55 for Lla244 and Lpl1524, respectively (**Table 1**). In the three co-cultures with the donor strain Efa2412, the final pH was significantly lower compared to that of the donor strain in monoculture, in particular with the two *L. plantarum* strains. Considering the donor strain Lla2125, only its co-culture with Lpl465 significantly decreased the final pH, when compared to the monoculture of the donor strain. Concerning the donor strain Lla244, the final pH did not differ in mono- and co-cultures.

2.4 The type of interactions modulates the carbohydrate consumption

The consumption of milk carbohydrates, i.e. lactose, and lupin carbohydrates, i.e. raffinose and sucrose are shown in **Figure 4**. Lactose was hydrolysed by all strains except Lla450, in

agreement with the results observed using API gallery (see material and method section). The consumption of lactose in the co-cultures with the donor strain Efa2412 significantly varied depending on the associated receiver strain. 72 % of lactose was consumed with Efa2412 alone, against 30%, 62 %, and 87%, in its co-culture with Lla450, Lpl465 and Lpl1524, respectively. Lactose consumption was less impacted by the co-cultures involving the donor strain Lla2125. There was significantly less lactose consumed when Lla2125 was co-cultured with Lla450 (81 % of lactose consumed in co-culture against 90% with Lla2125 in monoculture). No significant differences were observed in the co-cultures involving Lla2125 and the two *L. plantarum* strains. The co-cultures involving the donor strain Lla244 did not change the percentage of lactose consumed (65%).

Sucrose was only consumed by the strains Lla450 (82 %), Lpl465 (72 %) and Lla244 (30 %) in monocultures. Considering the results of the API gallery (see material and method section), a diminution of sucrose was also expected with Efa2412 but was not observed in monoculture. However, its association with Lla450, Lpl465 and Lpl1524 significantly modified sucrose consumption as 47 %, 34 % and 13 % were consumed in the respective co-cultures, showing the individual carbohydrate consumption by the strains. Likewise, the strain Lla2125 did not consume sucrose from the medium, but the association with Lla450, significantly increased sucrose consumption to 12 %. The co-cultures involving the donor strain Lla244 did not change the percentage of sucrose consumed.

Concerning raffinose, even if the three receiver strains were capable to hydrolyse this carbohydrate, as shown by the API gallery results, less than 8 % was consumed by these strains in monocultures. The only association that significantly increased raffinose consumption to 20 % was the co-culture of Efa2412 with Lpl1524.

230 **Table 2.** Volatile compounds identified in CDM after 24 h of fermentation at 30°C with the six LAB strains in mono and co-cultures and in
231 unfermented CDM used as controls (CDM TRYP and CDM PROT).

Compound (trivial name)	m/z	Ab.	Identification	Associated odor/flavor[a]	CAS n°	LRI	Max fold-change[b]	Culture associated to the maximal ratio culture:control	Origin
3-Methylbutanal	58	MBT	S, LRI, DB	Fruity	590-86-3	918	905 ± 15	Lla244 x Lla450	cata Leu
2,3-Butanedione (diacetyl)	42	D	LRI, DB	Buttery	431-03-8	975	39 ± 3	Lla244 x Lla450	metab C
2,3-Pentanedione	100	PD	LRI, DB	Buttery	600-14-6	1062	150 ± 23.4	Efa2412_Lla450	metab C
Hexanal	44	H	LRI, DB	Grassy	66-25-1	1080	0.06 ± 0.02	Lla2125	CDM
1-Propanol, 2-methyl	74	MPP	LRI, DB	Fusel	78-83-1	1114	3890 ± 240	Lla244 x Lpl465	cata Val
Heptanal	44	HP	LRI, DB	Green	111-71-7	1183	0.12 ± 0.06	Lla2125 x Lla450	CDM
2-Hydroxy-3-butanone (acetoin)	88	AC	LRI, DB	Buttery	513-86-0	1277	22211 ± 3014	Lla2125 x Lpl1524	metab C
2-Nonanone	58	NN	S, LRI, DB	Cheesy	821-55-6	1383	6.8 ± 0.4	Lla244_Lla450	cata FFA
Acetic acid	60	A	S, LRI, DB	Sour	64-19-7	1476	558 ± 82	Efa2412 x Lla450	metab C
Benzaldehyde	106	BZH	S, LRI, DB	Nutty	100-52-7	1535	3.3 ± 0.1	Efa2412_Lla450	cata Phe
2-Methylthiolan-3-one	60	MTL	LRI, DB	Sulfurous fruity berry	13679-85-1	1539	20.6 ± 2.6	Lla244	cata Met
Benzeneacetaldehyde	91	BZAH	LRI, DB	Honey	122-78-1	1635	223 ± 7	Lla244	cata Phe

232 Receiver strains Lla450, Lpl465 and Lpl1524 were grown on CDM TRYP, which corresponds to CDM PROT supplemented with 0.5 g/L of casein tryptone. The selection
233 criteria for the volatile compounds was a ratio culture/control >5 or < 0.1. Compound name according to IUPAC (International Union of Pure and Applied Chemistry)
234 nomenclature, CAS=Chemical Abstract Service registry number, S = Retention time and mass spectrum from standard, LRI = Linear retention index, DB = mass spectral data
235 library NIST, Ab = abbreviation, cata = catabolism, metab = metabolism, C = carbohydrate, FFA = free fatty acid. For strain codes see Table 4.
236 [a] flavor description according to thegoodscentscompany.com
237 [b] ratio culture:control

2.5 Volatile compound profiles in direct co-cultures

Among volatile compounds, twelve compounds exhibiting significant changes in concentration in mono- or co-cultures compared to the unfermented control samples (fold-change >5 or < 0.1) were identified (**Table 2**). They represented five chemical classes: five aldehydes, one sulphur-containing compound, four ketones, one acid and one alcohol and they derived from different pathways: five from amino acids catabolism, four from carbohydrate metabolism and one from free fatty acid catabolism (**Table 2**). Two linear aliphatic aldehydes, heptanal and hexanal, were 10-fold more concentrated in the control medium than in LAB cultures. Co-cultures did not contribute to a further decrease in the concentrations of hexanal and heptanal, compared to the monocultures of the donor strains. The ten other volatile compounds were produced by the LAB strains both in mono- and co-cultures. The highest fold-changes between cultures and controls varied from ~3 for benzaldehyde to ~20,000 for acetoin. The greatest fold-changes were observed with the donor strain Lla244 and/or the co-cultures involving the receiver strain Lla450 for most (10 out of 12) volatiles (**Table 2**). Although the volatile profiles differed according to the donor and receiver strains, most of the compounds were produced in various amounts by all strains, except 2,3-pentanedione, only produced by two strains (Lla450 and Lla244).

The global profiles of volatile compounds produced mainly depended on the donor strains, as shown on the radar plots in Figure 5A, which illustrates the fold-change in log of the most impacted volatile compounds in mono- and co-cultures. The donor strains presented differences in the volatiles they produced in monocultures. Globally, Lla2125 produced the lowest level of volatiles and Lla244 the highest, especially benzeneacetaldehyde (BZAH), 2-methyl-1-propanol (MPP) and 2,3-pentanedione (PD). Efa2412 produced more benzaldehyde (BZH) and acetic acid (A) compared to Lla2125.

The association of Efa2412 with each receiver strain led to higher levels of five volatiles (PD, NN, BZH, BZAH (not shown) and MTL) compared to the monoculture of Efa2412 (**Figure 5B**). PD, NN, BZH, BZAH and MTL were associated with buttery, cheesy, honey, nutty and sulfurous fruity berry flavors, respectively. Concerning the donor strain Lla2125, its co-culture with the receiver strains significantly increased the production of four volatiles (D, NN, AC and MTL). D and AC were associated with buttery flavor. Concerning Lla244 and its co-cultures, there was no significant differences in the volatile compound produced (**Figure 5**).

2.6 Insight on the NH_2-containing compounds available for the receiver strains

Each donor strain showed a particular amino acid profile which could influence the growth of the receiver strains. Regarding the FAA released, we focused on the 13 considered as essential for the growth of *L. lactis* (19) and/or *L. plantarum* (20) and compared their content in the medium after culture of each of the donor strain and in the medium used to grow the receiver strains (CDM TRYP) (**Table 3**). Four FFA were exhausted in the medium after some cultures. Hence, there was no Met and Arg left after the culture of the three donor strains, while both these FAA were present in CDM TRYP. In addition, Efa2412 did not leave Tyr, while the two other donor strains did not leave Trp and Lla244 did not leave Ile. The concentrations of the FAA present also varied according to the donor strain: Efa2412 monoculture contained significantly more Val, His, Ile, Leu, Phe, and Trp compared to the two other donor strain monocultures and CDM TRYP; Lla2125 monoculture was characterised by higher concentrations of Asp, Thr, Ser, Glu, and Tyr.

In four out of the six co-cultures with the donor strains Efa2412 or Lla2125, the overall amount of FAA was significantly lower compared to the one in the donor strain monocultures: -65 %, -9 % -11 %, and -8 % with Efa2412xLla450, Efa2412xLpl465,

286 Lla2125xLla450, and Lla2125xLpl465, respectively. Arg, Trp, and Ile were no longer
287 detectable after the culture of Lla450 with Efa2412 could thus have limited the growth of both
288 donor and receiver strains. The remaining essential FAA were detectable (> 1 mg/L) in all
289 nine co-cultures after fermentation, suggesting they were not limiting (data not shown).

290 The concentrations in peptides were significantly reduced in the co-cultures of the donor
291 strain Efa2412 with each receiver (-54 %, -16.8 %, and -19.3 %, with Lla450, Lpl465, and
292 Lpl1524, respectively) (data not shown). The ratio FAA:total NH_2 compounds tended to
293 decrease in the cultures involving Lla450 and, in contrast, to increase in the cultures involving
294 Lpl465 and Lpl1524, indicating that the receiver *L. lactis* strain tested preferentially
295 consumed amino acids, whereas the *L. plantarum* strains preferentially consumed peptides.

296 **Table 3.** Concentrations (mg/L) of the 13 essential amino acids after 24 h fermentation of the
297 CDM PROT by the donor strains Efa2412, Lla2125 and Lla244; and present in the CDM
298 TRYP prior to the culture with the receiver strains. Percentages represent the quantity of each
299 amino acid released from milk and/or lupin proteins of the CDM. For strain codes, see Table
300 4. The significantly highest concentrations for each amino acid are shown in bold.

	Efa2412	*Lla2125*	*Lla244*	*CMD TRYP*
Asparagine (Asn)	6.9 (1.7 %)	**11.0** (2.7 %)	4.7 (1.3 %)	1.9
Threonine (Thr)	4.8 (2.7 %)	**7.3** (4.2 %)	2.1 (1.2 %)	2.4
Serine (Ser)	2.3 (0.9 %)	**6.6** (2.7 %)	1.4 (0.6 %)	2.4
Glutamic acid (Glu)	64.7 (6.2 %)	**111.9** (10.7 %)	61.9 (5.9 %)	1.0
Valine (Val)	**13.1** (5.8 %)	5.9 (2.6 %)	1.7 (0.7 %)	5.1
Methionine (Met)	0	0	0	**3.9**
Histidine (His)	**7.4** (7.2 %)	5.4 (5.2 %)	5.3 (5.1 %)	1.5
Isoleucine (Ile)	**5.7** (2.8 %)	3.2 (1.6 %)	0	3.4
Leucine (Leu)	**33.5** (9.0 %)	10.0 (2.7 %)	2.7 (0.7 %)	22.0
Tyrosine (Tyr)	0	**13.9** (5.5 %)	10.8 (4.3 %)	2.7
Phenylalanine (Phe)	**24.5** (11.7 %)	8.8 (4.2 %)	8.7 (4.2 %)	12.0
Arginine (Arg)	0	0	0	**15.3**
Tryptophan (Trp)	**10.1** (ND)	0	0	0

301 **2.7 The absence of interaction is not due to growth inhibitor production nor isoleucine**
302 **deficiency**

303 Regarding the lack of interaction between the donor strain Lla244 and the receiver strains,
304 two hypotheses were made: either the production of a growth inhibitor, or a deficiency in
305 available Ile. **Figure 6** shows that all receiver strains reached 10^9 CFU/mL within 24 h of
306 culture in the CDM TRYP. The receiver strains grew in the culture supernatant of Lla244
307 supplemented with casein tryptone: bacterial counts exceeded 5.10^8 CFU/mL and were

similar to the bacterial counts observed in the CDM TRYP. This result suggests that no growth inhibitor was produced by the donor strain Lla244. The receiver strains grew but did not reach 10^8 CFU/mL when cultured in the culture supernatant of Lla244 supplemented with 5 mg/L of Ile. This result suggests that isoleucine was not the only limiting growth factor explaining why no interactions were observed between the donor strain Lla244 and the receiver strains.

2.8 Expression of the functional outputs by the different co-cultures summarised by PCA on the whole dataset

A total of 33 variables of four categories were selected to characterize the functional outputs of the three monocultures of donor strains and the nine co-cultures: two acidification parameters, consumption of three carbohydrates, volatile compounds (n=12), and the nitrogen composition of the medium after 24 h (including the content in each of the 13 amino acids known to be essential to LAB, global content in FAA, peptides, and the percentage of FAA). The first and second dimensions accounted for 43.6 % and 32.6 % of total variance, respectively (**Figure 7**). The three replicates of cultures appeared all co-localised, which underlines the good reproducibility of the experiments. In most cases, the donor strain was grouped with its three co-cultures, excepted in one case (Efa2412 associated with Lla450).

Three groups were distinguished by hierarchical clustering. Group 1 gathered Lla244 and its co-cultures and the co-culture Efa2412 with Lla450 and was characterized ($p<10^{-5}$) by high concentrations in most of the volatile compounds (BZH, HP, MTL, BZAH, MBT, H, MPP, and D), low FAA concentrations and more specifically Ile and Val and a higher sucrose consumption. Group 2 gathered Lla2125 and its co-cultures and was characterized ($p<10^{-5}$) by high concentrations in some amino acids (Glu, Thr, Asn, Ser) and AC, and low concentrations in some volatile compounds (A, BZH). Group 3 gathered Efa2412 and its two co-cultures with *L. plantarum* strains, and was characterized ($p<10^{-5}$) by high concentrations in branched-

chain (Leu, Val, Ile), aromatic (Phe, Trp) amino acids and His, and globally in free NH_2-containing compounds, more specifically peptides, low acidification rates, and low Tyr concentration.

Globally, co-cultures of the donor strains Lla2125 and Efa2412 showed lower scores on the first dimension than the corresponding monoculture of the donor strain, indicating that they contained less sucrose, more lactose, had a lower pH and contained less NH_2-containing compounds (FAA and peptides).

3 Discussion

This study aimed at promoting positive interactions based on nitrogen nutritional dependencies between LAB strains in co-culture. This was done in a context of designing starters of new (mixed) food fermentation, and seeking consequences of such positive interactions on the functional outputs of the fermented media.

McCully *et al*. (21) previously determined that a nitrogen starvation response is important for a stable coexistence between *Escherichia coli* and *Rhodopseudomonas palustris*. Thus, we first conceived a chemically-defined medium, called CDM PROT, which contained proteins as the sole nitrogen source, so as to control the nutritional interactions between strains and allow only proteolytic strains to grow. A similar strategy was used by Ponomarova *et al.* (11) to study the interactions between yeast and LAB strains. Selecting a medium supporting the growth of microorganisms in co-cultures, but not in monocultures allowed evidencing nutrient cross-feeding. While *S. cerevisiae* growth varied very little between monoculture and co-culture with LAB in the CDM used, both *L. lactis* and *L. plantarum* could grow only when co-cultured with the yeast, suggesting metabolic dependencies. In our study, we used a mix of milk and lupin proteins as sole nitrogen source, and a mix of lactose, sucrose, and raffinose as carbon sources, to mimic the content of a mix of milk and lupin. This medium thus both facilitated the study of LAB interactions, and provided some insight on the outputs that could be obtained by fermenting a more complex and mixed food matrix. As expected, only the three proteolytic strains grew in CDM PROT, whereas none of the three non-proteolytic strains tested did. The growth of the latter was restored by supplementing the medium with a casein hydrolysate, which demonstrates that the lack of available nitrogen nutrients was the only factor that prevented their growth in CDM PROT. In co-cultures of pairwise proteolytic and non-proteolytic strains, the former, qualified as donors, were then expected to provide the latter, qualified as receivers, with nitrogen nutrients.

365 We chose two complementary approaches to explore the interactions between donor and
366 receiver strains. First, co-cultures in compartmented chambers facilitated the enumeration of
367 each strain without needing to develop selective enumeration media, in particular in the case
368 of the *L. lactis/L. lactis* co-cultures. These chambers allowed to quickly establish whether
369 both strains grew, when they started to grow, and whether the interactions required or not a
370 physical contact between the strains. In their review on co-culture systems, Goers et al. (22)
371 suggested that such devices were excellent tools to explore cell-cell interactions. The kinetics
372 of diffusion of metabolites from one compartment to the other, e.g. nitrogen nutrients and
373 lactic acid, which are expected to enhance or inhibit the growth of the receiver strains (23),
374 did not seem to alter the donor/receiver interactions, since bacterial counts were identical after
375 14 h in compartmented chambers and in direct co-cultures. Secondly, direct co-cultures were
376 chosen to investigate the functional outputs that resulted from LAB metabolism, because they
377 are closer to food fermentation than co-cultures in compartmented chambers. Three main
378 outputs were targeted: the acidification rate, which should be quick enough for economic and
379 sanitary reasons, the consumption of lactose and raffinose, because these carbohydrates can
380 induce intestinal discomfort, and the production of aroma compounds susceptible to desirably
381 influence food flavor.

382 Three cases of interactions were observed, which depended only on the donor strains and not
383 on receiver strains tested. Firstly, co-cultured with the donor strain *E. faecalis* CIRM-
384 BIA2412 (Efa2412), the receiver strains quickly started to grow and reached high counts
385 (above 10^9 CFU/mL), showing strong interactions. Secondly, co-cultured with the donor
386 strain *L. lactis* NCDO2125 (Lla2125), the receivers grew but stayed below 10^9 CFU/mL,
387 suggesting weaker interactions. Thirdly, co-cultured with the donor strain *L. lactis* CIRM-
388 BIA244 (Lla244), none of the receivers grew, suggesting the absence of positive interactions.
389 Concerning donor strains, their maximal growth was similar in mono- and co-culture (**Figure**

3), implying that the positive interactions observed were commensalistic (8), *i.e.* that the fitness of receivers increased with no apparent cost or benefit for the donors. These differences of interactions could be explained by different factors. The proteolytic activity of the donors probably had likely the main impact in terms of quantity and nature of peptides and free amino acids released (**Figure 2**). This also could be balanced by the respective nitrogen nutritional requirements of donors and receivers, as well as their kinetics of growth that could also influence the interactions observed (**Figure 3**), as developed below.

Regarding the proteolytic activity, the donor strain Efa2412 could be qualified as a "model" donor strain compared to the two other donors, provided the receivers with high concentrations both in several free amino acids (Trp, Leu, Val, Phe, Ile, Arg) and in peptides (**Figures 2** and **7**). Among the three donors, Lla244 liberated the lowest amounts of nitrogen nutrients, but the concentration in FAA exceeded that of the control medium that contained 0.5 g/L casein hydrolysate and the concentration of peptides exceeded that produced by Lla2125. It was thus unexpected that receiver strains, which grew in the control medium and in the co-culture with Lla2125, did not grow in co-culture with Lla244. We first hypothesised that Lla244 would produce a growth inhibitor such as a bacteriocin, but this was invalidated by the results observed in sequential cultures, which showed that receiver strains grew in the culture supernatant of Lla244 supplemented with 0.5 g/L of casein hydrolysate (**Figure 6**). We also hypothesised that Ile concentration limited receiver growth since Ile was the only essential amino acid lacking in Lla244 monoculture (**Table 3**). This second hypothesis was also ruled out since Ile addition did not restore the growth of the two *L. plantarum* strains in direct co-culture with Lla244 (**Figure 6**). These results suggest that the nutritional dependencies based on nitrogen sources rely on both the nature and concentration of nitrogen nutrients.

414 The nutritional requirements and preferences for peptides or FAA of both donor and receiver
415 strains can also modulate their interactions. In our study, the comparison between FAA and
416 peptide uptakes by the receiver strains suggests that the *L. lactis* receiver had no significant
417 preference, whereas the two *L. plantarum* strains preferred peptides to FFA (data not shown).
418 Such a preference for peptides by a *L. plantarum* strain was demonstrated by Saguir *et al*. (24)
419 who observed that dipeptides were more effective than FAA in sustaining its growth under
420 nutritional stress conditions. This is also in agreement with a higher number of amino acids
421 required for the growth of the species *L. plantarum* compared to *L. lactis* and *E. faecalis* (20,
422 25, 26).

423 Finally, the growth kinetics of donor and receiver strains could also have influenced the
424 interactions observed in the present study. The two *L. plantarum* receivers grew slower than
425 the three donors (**Figure 3**) and thus could have been inhibited in co-culture by the lactic acid
426 early produced by the donor strain. However, they can support lower pH (27) and thus keep
427 growing even after the donor strain stopped growing. The amensal interactions observed
428 between Lla244 and the receiving strains may also be due to the fastest acidification and
429 lowest final pH obtained with this donor strain compared to the two others (Table 1). To
430 counteract such inhibitions, the inoculation ratios of the donors/receivers associated could be
431 adjusted by increasing the initial counts of the receiver, and/or the donor strains could be
432 chosen among strains that do not exhibit too high acidification rate nor reduce pH to too low
433 values, as it is the case for the donor Efa2412 compared to the donor Lla2125.

434 The stronger the interactions, the more the outputs observed in co-cultures differed from the
435 ones observed in monoculture of the donor strains. In co-cultures with the donor strain
436 Efa2412, which induced the strongest interactions, all the functional outputs characterised in
437 this study were impacted: the rate and degree of acidification, the consumption of three

carbohydrates and the production of some aroma compounds. When compared to the monoculture Efa2412, a lower final pH was observed in the co-cultures with the two *L. plantarum* receivers, a higher acidification rate with Lla450 receiver, and lower final concentrations in lactose, sucrose, and/or raffinose, depending on the receivers. The maximum consumption of raffinose was only 20% in MCD media, although the three receiver strains were capable of using it, as shown with the API 50CHL gallery results (Table 4). This apparent discrepancy most likely results from preferences between the three carbohydrates contained in CDM, i.e. lactose, sucrose and raffinose. In agreement with our results, lactose and/or sucrose were preferentially used, compared to raffinose, by nearly 300 LAB strains of 25 different species grown in soy juice (28). Concerning sucrose and lactose preference, Efa2412 and Lpl1524 preferred sucrose over lactose, whereas Lpl465 preferred lactose over sucrose. The volatile profile was also markedly impacted in the co-cultures with the donor Efa2412, with five out of the 12 aroma compounds significantly more produced, associated with varied potentially desirable aroma: acetoin (milky), 2-nonanone (fruity-cheesy), 2,3-pentanedione (sweet-caramel-buttery), benzaldehyde (almond) and 2-methylthiolan-3-one (fruity). In co-cultures with the second donor strain, Lla2125, which induced only weak interactions, smaller impacts on the functional outputs were observed. When compared to the monoculture of Lla2125, a lower final pH was observed in the co-cultures of the receivers Lpl465, and four out of the 12 aroma compounds were produced in higher concentrations: 2-nonanone (fruity-cheesy), 2,3-pentanedione (sweet-caramel-buttery), 2-methylthiolan-3-one (fruity) and diacetyl (buttery). Finally, in co-cultures with the donor strain Lla244, in which there was no interaction, no further functional outputs were observed compared to the monoculture of this strain.

Many LAB properties are strain-dependent (29), and consequently so are the changes in the functional outputs observed. This gives great opportunity to choose receivers able to modulate

463 the functional outputs targeted. In our study, for example, the growth of the receiver Lla450
464 increased the concentration of some desirable aroma compounds such as 2,3-pentanedione
465 and 2-methylthiolan-3-one in co-culture. The two *L. plantarum* receivers led to a lower final
466 pH, while Lpl1524 receiver was associated with a decrease in raffinose content. Although
467 residual raffinose remained in the medium, the raffinose-hydrolysis activity of Lpl1524 (α-
468 galactosidase) may also remain active in the intestine, as observed for lactase activity (β-
469 galactosidase) after yogurt ingestion (30). In conclusion, our attempt to enforce nitrogen-
470 based nutritional dependencies between LAB strains did not necessarily ensure positive
471 interactions. The resulting functional outputs of fermented media depend on the strength of
472 the interactions binding the LAB strains in co-culture. We showed that the amount and the
473 nature of the FFA and peptides released by donor strains impacted the growth of receivers.
474 Further investigation of the peptides produced and consumed would be required to better
475 understand the interactions observed in this study. Omic studies such as transcriptomics,
476 proteomics, peptidomics, and/or metabolomics could also be of great interest to investigate
477 the mechanisms of the interactions observed (31, 32). Genetic insight could also be useful to
478 manipulate the genomes to confirm the mechanisms observed. In regard to better understand
479 complex microbial communities, future studies are required, including an increased genetic
480 diversity, by adding multiple strains of different genera and species.

481 **4 Materials and Methods**

482 **4.1 Bacterial collection**

483 Six mesophilic LAB strains were tested in the following experiments: two *Lactiplantibacillus*
484 *plantarum*, one *Lactococcus lactis* and one *Enterococcus faecalis* strains belonging to the
485 collection of CIRM-BIA: International Centre for Microbial Resources dedicated to bacteria
486 of food interest, (INRAE Rennes, France, https://www6.rennes.inrae.fr/stlo_eng/), and two *L.*
487 *lactis* strains from NCDO: National Collection of Dairy Organisms; now NC of Food

Bacteria, (Berkshire, UK). The strains were selected on their proteolytic capacities in order to either obtain donor strains able to furnish peptides from the medium, and receiver strains unable to grow without an external source of peptides or free amino acids.

Table 4. Origin and characteristics of the strains used for proteolytic activity and carbohydrate consumption using API 50CHL™ galleries.

Genus	*Species*	Strain number	Origin	Strain code	PI (%)	LAC	SUC	RAF
Enterococcus	*faecalis*	CIRM-BIA2412	NA	Efa2412	10.8	+	+	-
Lactococcus	*lactis*	NCDO2125	Termite Gut	Lla2125	5.5	+	-	-
Lactococcus	*lactis*	CIRM-BIA244	Raw milk	Lla244	3.6	+	+	-
Lactococcus	*lactis*	NCDO2111	Pea	Lla450	0	-	+	+
Lactiplantibacillus	*plantarum*	CIRM-BIA465	Sauerkraut	Lpl465	0	+	+	+
Lactiplantibacillus	*plantarum*	CIRM-BIA1524	Silage	Lpl1524	0	+	+/-	+

LAC = lactose, SUC = sucrose, RAF = Raffinose. PI = proteolytic indices, determined after 24 h of culture in a chemically defined medium containing a mix of caseins and lupin proteins, expressed as a percentage of free NH_2-containing compounds liberated relative to the calculated maximal amino groups that can be released. NA = non-available data.

4.2 Composition of the chemically defined medium

The chemically defined medium (CDM) was developed in order to fulfil all the nutritional requirements of lactic acid bacteria (mainly lactococci and lactiplantibacilli) (33) in terms of vitamins, mineral salts and nucleic acids. The nitrogen source was solely in the form of protein thus limiting access to amino acids to proteolytic strains. The protein and carbon resources were chosen to mimic the composition of both resources: lactose and caseins for milk part, sucrose, raffinose and lupin proteins for the legume part.

The final composition of the medium used was the following:

Buffer: K_2HPO_4/KH_2PO_4: 50 mM, pH 6.9 ± 0.1 ; Sugars: Lactose$^{(2)}$: 5 g/L, sucrose$^{(2)}$: 5 g/L, raffinose$^{(2)}$: 5 g/L ; Nitrogen source: sodium caseinate (Eurial, Nantes, France): 2,5 g/L, lupin protein isolate (homemade LPI, obtained from Protilup 450 flour (Lup'ingrédient, Martigne-Ferchaud, France) by precipitating the proteins at pH 4,6 (15) 2,5 g/L ; Mineral salts: $CaCl_2$$^{(1)}$: 25 mg/L, $Cl_2Co$$^{(2)}$: 1 mg/L, $CuCl_2*2H_2O$$^{(1)}$: 4 mg/L, $MgCl_2*6H_2O$$^{(?)}$: 25 mg/L,

510 $(NH_4)_6Mo_7O_{24}*4H_2O$[(1)]: 1 mg/L, $ZnSO_4*7H_2O$[(2)]: 1 mg/L, $MnSO_4$[(1)]: 100 mg/L, $FeSO_4$[(1)]: 5
511 mg/L ; Vitamins: Riboflavin[(2)]: 3 mg/L, Nicotinic acid[(2)]: 3 mg/L, Ca-pantothenate[(2)]: 3 mg/L,
512 Pyridoxine[(2)]: 1 mg/L, Biotin[(2)]: 0,5 mg/L, Folic acid[(2)]: 1 mg/L, Thiamine, HCl[(2)]: 0,5 mg/L,
513 p-aminobenzoic acid[(2)]: 1 mg/L, Pyridoxal, HCl[(2)]: 1 mg/L ; Nucleic acids: Adenosine[(2)] 10
514 mg/L, Guanine[(2)]: 10 mg/L, Uracil[(2)]: 10 mg/L, Inosine[(2)]: 10 mg/L, Orotic acid[(2)]: 10 mg/L,
515 Thymidine[(2)]: 10 mg/L ; Fat: Tween 80[(2)] : 0,5 g/L, provided by [(1)] Merck, Darmstadt,
516 Germany and [(2)] Sigma - Aldrich, Munich, Germany.

517 The protein fraction was prepared separately from the other constituents. A twofold
518 concentrated solution of lupin proteins and caseins was prepared in osmosed water and
519 sterilized by autoclaving at 115°C during 20 min. In parallel, a twofold concentrated solution
520 of vitamins, mineral salts, sugars, Tween 80 and buffers were sterilized by filtration through a
521 0.2 µm PES membrane filter (Thermo Scientific, Waltham, Massachusetts, USA). Then these
522 two solutions were mixed together to constitute the CDM PROT, which was stored at 4 °C for
523 less than 1 month and isolated from the light with aluminium foil.

524 A supplementary CDM, referred to as CDM TRYP was also prepared to enable the growth of
525 the non-proteolytic strains. It consists in the same CDM preparation, supplemented with 0.5
526 g/L of casein tryptone (Biokar, Allonne, France). The receiver strains were always grown in
527 the CDM TRYP when tested in monocultures.

528 Two media were used to verify the absence of growth inhibitor in *Lactococcus lactis* CIRM-
529 BIA244 cultures and to verify the deficiency in isoleucine in these cultures. The two media
530 first consisted in the culture supernatant of *L. lactis* CIRM-BIA244 in CDM PROT incubated
531 for 24 h at 30 °C, harvested by centrifugation at 5000 g x 10 min and adjusted at pH 7 with
532 NaOH 5 N. The supernatant was sterilized using a 0.22 µm filter. For the medium used for the
533 verification of the absence of growth inhibitor, a sterile 10 % tryptone solution was added to

reach a final concentration of 0.5 g/L. For the medium used for the verification of the deficiency of isoleucine, a sterile solution of isoleucine 10 % was added to reach a final concentration of 5 mg/L

4.3 Culture conditions

4.3.1 Pre-cultures

LAB strains were conserved in cryotubes at -80 °C. One cryotube was used for each replicate culture. Bacteria were cultured once in a rich medium: M17 for lactococci (34) and de Man Rogosa and Sharpe broth (MRS) for lactobacilli (35). Two pre-cultures were made in the CDM PROT in order to adapt the proteolytic strains to the medium. The receiver strains that are not proteolytic were cultured on CDM TRYP to enable their growth.

4.3.2 Compartmented chambers set-up

The compartmented chambers (C.P.I.L, Issoire, France), similar to the one used by Paul et al. (36) had a usable volume of 25 mL (30 mL total) and were fixed together with a clamp. O-rings were placed between the two chambers so as to guaranty the sealing of the system. The membrane used to separate the two compartments was a 0.4 µm polycarbonate membrane (Isopore^{TM}, Merck Millipore, Darmstadt, Germany). Prior to inoculation, the chambers as well as the filters were sterilised at 121°C for 15 min. O-rings were decontaminated using ethanol and rinsed with sterile deionised water. The whole set-up was installed under sterile conditions.

4.3.3 Co-cultures in compartmented chambers and direct co-cultures

The three proteolytic strains: Lla244, Lla2125, Efa2412, referred to as donors, were associated in direct co-cultures or in compartmented chambers, each with each of the three non-proteolytic strains: Lla450, Lpl465 and Lpl1524, referred to as receivers, thus generating

nine pairs of donor/receiver strains. In all cases, the strains were then inoculated at a total count of 10^7 colony-forming units (CFU) /mL of CDM PROT or CDM TRYP, either in chambers (25 ml in each chamber) or in Falcon tubes of 15 mL. One falcon per time and several chambers were used so that volumes would not be limiting for the sampling (up to 6 samples of 2 mL were taken per chambers).

In the chambers, the strains were incubated at 30°C for 24 h, with a low orbital shaking of 65 rpm in order to limit the medium aeration, while improving diffusion. The CDM were also incubated as an unfermented control.

Culturable bacterial counts were determined with appropriate diluted suspensions of the samples in 1 g/L tryptone + 8.5 g/L NaCl solution in micro-plates (37). Lactococci and enterococci were incubated for 24 to 48 h under aerobic conditions in M17-glucose, and lactiplantibacilli were cultured for 48 h anaerobically using CO_2 generators (BD Biosciences, San Jose, USA) in MRS, both at 30°C.

Direct co-cultures were incubated at 30°C for 24 h, and samples were then collected for further carbohydrate and volatile analyses. The growth of donor strains was controlled after reaching their maximum population, i.e. after ~ 14 h of culture in both mono and co-cultures using a selective medium constituted by the CDM PROT medium added with 12 g/L agar, incubated for 72 h. Cultures were made in triplicates, independently.

4.4 Biochemical analyses

4.4.1 Acidification parameters

Acidification kinetics were established in direct co-cultures, using a wireless iCINAC (AMS, Frépillon, France) to estimate the maximal acidification rates by calculating the slope between pH 5.5 and pH 5 in all graphs. The final pH was measured using a pHmeter (WTW, Weilheim, Germany) after 24 h of incubation.

581 **4.4.2 Carbohydrate analysis**

582 100 µL of sulfosalicylic acid 2.3 M were added to 1 mL of sample for deproteinization. The
583 samples were then placed at 4°C for 1 h prior to centrifugation at 10,000 g for 15 min. The
584 Supernatants were then filtered through a 0.22 µm membrane and stored at -20°C prior to
585 analyses. Lactose, sucrose, raffinose, were quantified by anion exchange chromatography
586 ICS-5000+ Dionex (Thermo Electron SA Courtaboeuf France) fitted with a CarboPac PA210-
587 4µm (2 × 150 mm) analytical column (preceded by a corresponding guard column 2 × 30
588 mm). The eluent used was KOH generated with the eluent source Dionex EGC 500 KOH+
589 Eluent Generator Cartridge and ultrapure water from arium® pro system, Sartorius. HPLC-
590 EC was run at 30 °C with a the flow rate of 0.2 ml/min and the gradient was as follows: initial
591 conditions 13 mM of KOH maintained for 32 min then a linear rise to 42 min up to 100 mM
592 KOH maintained from 42 to 52 min followed by reversion to the initial conditions with a
593 linear decrease from 52 to 60 min. Quantification was performed with an external calibrating
594 using carbohydrate standards (Sigma-Aldrich) prepared at 2, 5, 10, 20 and 40 mg/L (linearity
595 range).

596 **4.4.3 Volatile compound analysis**

597 Volatile compounds were extracted using a Turbomatrix HS-40 trap automatic headspace
598 sampler and analysed using a Clarus 680 gas chromatograph coupled to Clarus 600T
599 quadrupole mass spectrometer, operated within a mass range of m/z 29-206 and ionisation
600 impact of 70 eV (PerkinElmer, Courtaboeuf, France) as detailed in (38). In brief, 2.5 g of
601 samples were placed in 20 mL PerkinElmer vial and stored at -80°C prior to analysis.
602 Compounds were eluted on an Elite WAX ETR column (30 m × 0.25 mm × 0.25 µm;
603 PerkinElmer, Waltham, MA, USA), with helium as the mobile phase, in the following
604 conditions: initial temperature 35°C maintained for 10 min, then increase at 5°C/min up to

230°C. Volatile compounds were identified using the NIST 2008 Mass Spectral Library data (Scientific Instrument Services, Ringoes, NJ, USA) and by comparing the retention indexes and mass spectral data of standards. Volatiles were semi-quantified from the abundance of one specific mass fragment (m/z), in arbitrary units. MS data were processed using XCMS on the R software (39). The full width at half maximum was set to 5, the maximum number of peaks per ion to 100, the interval of m/z value for peak picking was set to 0.4, the signal to noise ratio threshold to 6, the group band-width to 3 and the minimum to 0.4. The other parameters were those by default.The results are expressed as fold-change, i.e. ratio between their concentration in cultures and in unfermented CDM used as controls.

4.4.4 Amino acids analysis

Free amino acid content was determined after deproteinization as described for the carbohydrate analyses (2.4.2 material and methods section). After filtration through a 0.45 µm pore size membrane (Sartorius, Palaiseau, France), the supernatants were diluted three times with 0.2 M lithium citrate buffer (pH 2.2) prior to injection. Amino acids were analysed using cation exchange chromatography on a Biochrom 30 AA analyser (Biochrom Ltd, Cambridge, UK) according to Spackman et al. (40) with lithium citrate buffers as eluents and the ninhydrin as a post-column reaction system.

Total amino acid content in the LPI and caseinate was determined with a complete hydrolysis of the proteins with concentrated HCl. These samples were hydrolyzed at 110°C for 24 h in presence of 6 N HCl using 1.5 mL acid for an equivalent of 2 mg of total nitrogen. Tubes were then dried and samples were re-suspended in 5 mL of the 0.2 M lithium citrate buffer (pH 2.2) prior to injection on the Biochrom 30 AA analyser. For sulfurous amino acids, samples were initially oxidized overnight at 0°C with 2 mL of performic acid then dried before the acid hydrolysis.

4.4.5 Free amino groups dosage and proteolytic indices calculation

The changes in the amount of nitrogen compounds, i.e. peptides and free amino acids present in the CDM fermented or not after 24 h of incubation were measured in triplicates using the o-phtaldialdehyde (OPA) method of Church et al. (41) adapted to microplate. The proteins were precipitated prior to the assay by half-diluting samples with 2% (w/w) trichloroacetic acid final concentration for allowing the free NH_2 groups present at the N-terminal extremity of the peptides and amino acids to be preferentially detected by the OPA. The results were expressed as mg of free NH_2 / mL. Methionine was used as a standard.

The proteolytic indices (PI), represents the number of free amino groups relative to the total amino groups. It is therefore available calculating the ratio of OPA response of fermented samples (OPA_{sample}) relative to that of acid hydrolysates OPA_{max}, as follows:

$$PI = OPA_{sample}/OPA_{max}\text{ , expressed in \%}$$

The quantified FAA and the overall NH_2 group values were used to calculate the content in peptides by difference between the total NH_2 values and the FAA values, converted in NH_2 content.

4.5 Statistical analyses

Analyses of variance (ANOVA) were performed to determine whether the acidification and growth parameters, the carbohydrate and volatile contents differed according to the mode of culture used (mono or co-culture), using the function aov of R (R version 3.5.1 (2018-07-02), RStudio, Inc.). Means were compared using the Tukey posthoc test from the package car of R (p-value < 0.05). ANOVA for the acidification parameters and carbohydrates hydrolysis were made on the whole dataset gathering the three monocultures of donor strains, the three monocultures of the receiver strain and the nine co-cultures. For volatiles, ANOVA were

made on the ratio of each compounds compared to the control (fold-change) within four data subsets: Efa2412 mono- and co-cultures; Lla2125 monoculture and co-cultures; Lla244 mono- and co-cultures; the three receiver strains grown in CDM TRYP.

Principal component analyses (PCA) and a hierarchical clustering on principle components (HCPC) were performed using the FactoExtra package of R.

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780

7 Figures and captions

Figure 1. Schematic representation of the strategy used to favor positive interactions between two lactic acid bacteria strains.

A first strain is selected for its proteolytic activity and its capacity to produce the output A (*e.g.* aroma compounds). Its protease(s) will degrade the proteins present in the medium into peptides and free amino acids. The first strain is hence referred to as « donor ». A second strain is selected for its capacity to produce the output B (*e.g.* carbohydrates hydrolysis), its lack of proteolytic capacity and its amino acid auxotrophies. It will then benefit from the pool of peptides and free amino acids available, and is hence referred to as « receiver ». The objective is to favor commensalism or mutualism between the two strains in co-culture.

Figure 2. Donor strains were selected for their distinct proteolytic profiles

Concentrations of free NH_2 groups (mg/mL) after 24 h fermentation of the CDM PROT by the donor strains Efa2412, Lla2125 and Lla244, and those present in the CDM TRYP and CDM PROT prior to culture. The proteolytic indices (PI) obtained for each donor strain cultured in the CDM PROT was calculated as the ratio of the peptides and free amino acids (FAA) measurable by OPA during fermentation (OPA_{sample}) and the OPA_{max}. The total amount of free NH_2 has been separated into the part related to free amino acids and the one related to peptides, calculated by difference between total and free amino acid-related NH_2 groups, converted in OPA response using the free amino acids dosage. For strain code, see Table 4.

Figure 3. Three different donor stains resulted in three different types of interactions

Growth curves of the six LAB strains used in the study incubated in compartmented chambers at 30 °C for 24 h.

806 The proteolytic (donor) strains (bottom): *Enterococcus faecalis* CIRM-BIA2412 (Efa2412, ,
807 full purple squares), *Lactococcus lactis* NCDO2125 (Lla2125, , blue circles) and *L. lactis*
808 CIRM-BIA244 (Lla244, , orange triangles),
809 The non-proteolytic (receiver) strains (top): *L. lactis* NCDO2111 (Lla450, orange stars),
810 *Lactiplantibacillus plantarum* CIRM-BIA465 (Lpl465, empty green squares) and *L.*
811 *plantarum* CIRM-BIA1524 (Lpl1524, red diamonds), in mono and co-cultures. Each one of
812 the proteolytic strains was associated with one non-proteolytic strain. Bacterial counts were
813 made on MRS agar for *L. plantarum* strains and M17 for the rest of the strains.
814

815 **Figure 4. Carbohydrates consumption can vary according to the strains associated**

816 Percentages of lactose, sucrose and raffinose consumed in mono- and co-cultures of LAB
817 donor and receiver strains during 24 h incubation at 30°C. Percentages were calculated as a
818 ratio of the amount in the carbohydrate after fermentation relative to the amount of the non-
819 inoculated media used as a control: CDM PROT was used as the control for the donor strains
820 Efa2412, Lla2125, Lla244 and the co-cultures, and CDM TRYP for the receiver strains
821 Lla450, Lpl465 and Lpl1524 monocultures. Initial concentrations in g/L: [lactose] = 4.78 ±
822 0.50, [sucrose] = 4.03 ± 0.21, [raffinose] = 4.63 ± 0.23.

823 **Figure 5. Co-cultures increased the concentrations of volatile compounds potentially**
824 **associated with pleasant flavors**

825 A) Radar charts of the abundance ratio (culture:control) of the 12 volatile compounds
826 selected, expressed in log for the LAB donor strains Efa2412 and its co-cultures, Lla2125 and
827 its co-cultures, and Lla244 and its co-cultures, all cultured in CDM PROT. Ø represents the
828 absence of the receiving strain, *i.e.* the monocultures of the donor strains. The * represents the
829 volatile compounds that are significantly increased in at least one co-culture compared to the

donor strain in monoculture. B) Fold changes (culture:control) of 6 volatile compounds whose concentrations were significantly increased with co-cultures. For the strain codes, see Table 4. H = hexanal, HP= heptanal, MBT= 3-methylbutanal, BZH = benzaldehyde, BZAH = benzene acetaldehyde, MTL = 2-methylthiolan-3-one, D = diacetyl, PD = 2,3-pentanedione, AC = acetoin, NN = 2-nonanone, A = acetic acid, MPP = 2-methyl-1-propanol.

Figure 6. The absence of interaction is not related to a growth inhibitor factor nor a deficiency in isoleucine

Bacterial counts of the three receiver strains cultured in CDM + TRYP: CDM PROT supplemented with 0.5 g/L of casein tryptone; (Lla244) +TRYP: supernatant of the donor strain Lla244 cultured in CDM PROT for 24 h, and supplemented with 0.5 g/L of casein tryptone; (Lla244) + Ile: supernatant of the donor strain Lla244 cultured in CDM PROT for 24 h, and supplemented with 5 mg/L of isoleucine.

Figure 7. Parameters differentiating the cultures

Biplot of the first two dimensions of principal component analysis performed using 32 variables: 12 selected volatile compounds expressed as a culture:control (non-inoculated CDM PROT) ratio, two acidification parameters: maximal acidification rate (Vmax) and final pH, three carbohydrates, expressed as the percentage of carbohydrate consumed (LAC, SUC, RAF), and 15 variables that describe the content in free amino acids and peptides: 13 essential amino acids, free NH_2 groups (NH2), concentration in free amino acids (FAA), concentration in peptides (PEPT) measured in CDM PROT fermented by the monocultures of the strains Efa2412, Lla2125 and Lla244 and their co-cultures with three receiver strains Lla450, Lpl465, Lpl1524. Replicate experiments are represented using the same symbols. For compounds abbreviation see Table 2. The ellipses result from a hierarchical clustering

performed on the PCA dataset. For strain codes, see Table 4. H = hexanal, HP= heptanal, MBT= 3-methylbutanal, BZH = benzaldehyde, BZAH = benzene acetaldehyde, MTL = 2-methylthiolan-3-one, D = diacetyl, PD = 2,3-pentanedione, AC = acetoin, NN = 2-nonanone, A = acetic acid, MPP = 2-methyl-1-propanol.