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The Fatty-Acid Hydratase Activity of the Most Common Probiotic Microorganisms

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Abstract: In this work, we studied the biotechnological potential of thirteen probiotic microorganisms currently used to improve human health. We discovered that the majority of the investigated bacteria are able to catalyze the hydration reaction of the unsaturated fatty acids (UFAs). We evaluated their biocatalytic activity toward the three most common vegetable UFAs, namely oleic, linoleic, and linolenic acids. The whole-cell biotransformation experiments were performed using a fatty acid concentration of 3 g/L in anaerobic conditions. Through these means, we assessed that the main part of the investigated strains catalyzed the hydration reaction of UFAs with very high regio- and stereoselectivity. Our biotransformation reactions afforded almost exclusively 10-hydroxy fatty acid derivatives with the single exception of *Lactobacillus acidophilus* ATCC SD5212, which converted linoleic acid in a mixture of 13-hydroxy and 10-hydroxy derivatives. Oleic, linoleic, and linolenic acids were transformed into (R)-10-hydroxystearic acid, (S)-(12Z)-10-hydroxy-octadecenoic, and (S)-(12Z,15Z)-10-hydroxy-octadecadienoic acids, respectively, usually with very high enantiomeric purity (ee > 95%). It is worth noting that the biocatalytic capabilities of the thirteen investigated strains may change considerably from each other, both in terms of activity, stereoselectivity, and transformation yields. *Lactobacillus rhamnosus* ATCC 53103 and *Lactobacillus plantarum* 299 V proved to be the most versatile, being able to efficiently and selectively hydrate all three investigated fatty acids.

Keywords: probiotics; biocatalysis; hydratase; oleic acid; linoleic acid; linolenic acid; hydroxy fatty acids; stereoselective biotransformations; whole-cell processes

1. Introduction

Probiotics are defined as live microorganisms intended to provide health benefits when administered in adequate amount, generally by improving or restoring the gut flora. Being regarded as beneficial for human health, they are included in the list of the GRAS microorganisms (Generally Recognized As Safe) and have gained increasing scientific and industrial relevance during the last few decades [1,2]. The growth of the probiotics market has fostered the biochemical studies of these species, especially those regarding the microorganism–human being interaction. Despite this fact, their use as biocatalysts for industrial biotransformation is restricted to a limited number of applications [3–12]. Among the most known probiotic bacteria, those belonging to the genus *Lactobacillus* and *Bifidobacterium* turned out to be the most promising for industrial purposes. In this context, the biotransformation processes involving the Δ^{9-10} double bond hydration of the C_{18} unsaturated fatty acids have received increasing attention. In fact, oleic, linoleic, and linolenic acid (Figure 1) are the main components (as triglycerides) of the vegetable oils used for human consumption.

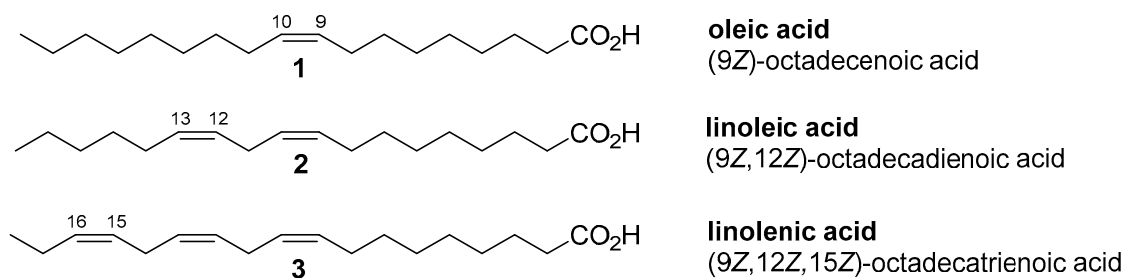


Figure 1. The most common C₁₈ unsaturated fatty acids: oleic (1), linoleic (2), and linolenic (3) acid.

These fatty acids share in common the presence of a *cis* double bond between C(9) and C(10) whose hydration, in principle, can afford both 9-hydroxy-fatty acids (9-HFAs) and 10-hydroxy-fatty acid (10-HFA) derivatives. Although the aforementioned positions of the fatty acid chain possess very similar chemical reactivity, the main part of the hydrating bacteria exclusively provides the 10-hydroxy-derivatives, even if the transformed FAs contain other unsaturations. In addition, this kind of biotransformation is usually very stereoselective and a single enantiomer is produced.

This biochemical process has been studied since the early 1960s [13], but the class of enzymes responsible for the hydration step have been characterized only recently [14]. These enzymes are collectively classified as oleate hydratases (EC 4.2.1.53) and have received growing attention both from chemists and biologists [15]. A number of putative oleate hydratases have been identified from bacteria including different probiotic strains, and then isolated and characterized by overexpression in heterologous hosts [16,17].

HFAs are important chemicals widely used for many industrial applications such as starting materials for biodegradable polymers, lubricants, emulsifiers, drugs, cosmetic ingredients, and flavors [18–21]. It is worth noting that HFAs and some of their derivatives also possess relevant biological activities. A mixture of 10-HFAs was patented as an intestinal tract protecting agent [22] while 10-hydroxystearic acid proved to ameliorate skin age spots and conspicuous pores [23]. Fatty acid esters of 13-hydroxy linoleic acid are anti-inflammatory [24]; 9-hydroxystearic acid (9-HSA) shows anticancer properties [25] whereas (12Z)-10-hydroxy-octadecenoic acid possesses antifungal properties [26] and is able to prevent gastric *Helicobacter* infections by blocking their futasine pathways [27]. In addition, recent studies have demonstrated that HFAs produced by human gut bacteria play a relevant role in the control of allergy, inflammation, and immunity, pointing to the involvement of their metabolites in the so-called gut–skin axis [28,29].

Despite the general relevance of HFAs and the specific interest related to their effect on human health, the hydratase activity of the probiotics toward the three most common unsaturated fatty acids (UFAs) 1–3 have been explored only marginally. More specifically, the probiotics-mediated biotransformations of UFAs have been tested, mainly using oleic and linoleic acid as substrates, whereas a limited number of studies have examined the biotransformation of linolenic acid [12,30]. Furthermore, the activity of the hydratases has been investigated, mainly making use of recombinant hydratases and thus employing isolated enzymes instead of studying the fatty acid biotransformation in whole-cell processes. This matter is very relevant as any given probiotic microorganism might express more than a single hydratase as well as further transform the obtained HFAs by oxidation, elimination or esterification reactions.

Overall, the whole-cell hydration-based biotransformations that can be performed using oleic, linoleic, and linolenic acids as substrates are summarized in Figure 2. Even if these reactions have been accomplished using either probiotic or non-probiotic microorganisms [31–37], recent studies have proven that the gut lactic acid bacteria possess the enzymatic machinery necessary to perform all the described biotransformations [29,38]. In addition, a number of 10- and 13-hydratases have been cloned from probiotic bacteria and have been employed for the synthesis of a number of HFA derivatives [16].

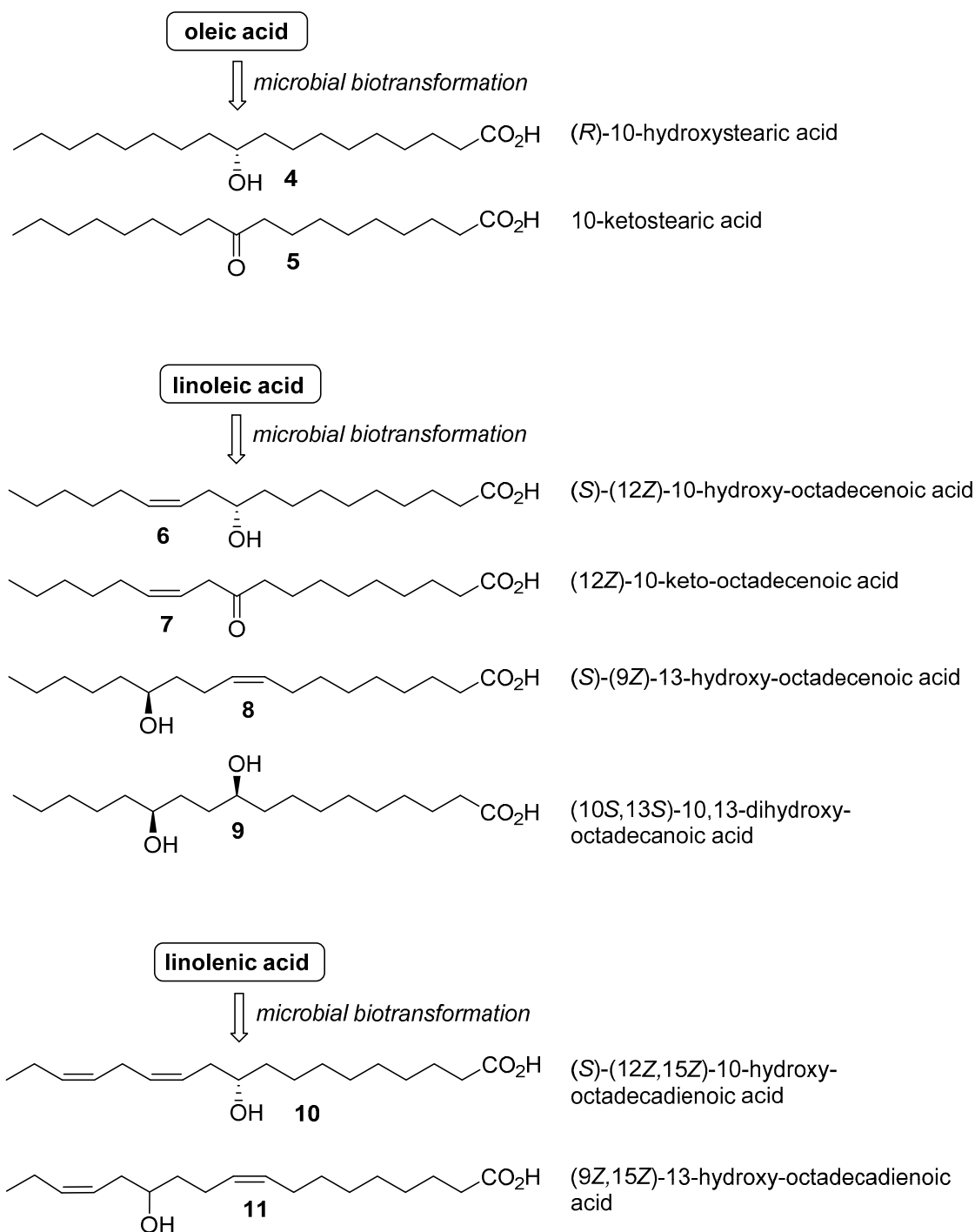


Figure 2. The hydration-based biotransformations of the oleic (1), linoleic (2), and linolenic (3) acids, potentially mediated by probiotic bacteria.

As stated previously, HFAs are usually formed with high regio- and stereoselectivity. According to our recent work [12], the widely used probiotic *Lactobacillus rhamnosus* LGG converts acids 1–3 into the corresponding 10-hydroxy derivatives, namely (*R*)-10-hydroxystearic acid (4), (*S*)-(12*Z*)-10-hydroxy-octadecenoic acid (6), and (*S*)-(12*Z*,15*Z*)-10-hydroxy-octadecadienoic acid (10), respectively, in very high enantiomeric purity (ee > 95%). A number of other probiotics possess similar biocatalytic properties with high selectivity toward the hydration of position 10 of the fatty acid chain. Nevertheless, only few relevant exceptions to this general trend have been reported. An important example concerns

some strains of *Lactobacillus acidophilus* [39–41] and *Lactobacillus plantarum* [42] that possess both 10- and 13-hydratase activity, most likely due to the simultaneous activity of two different enzymes. Therefore, the whole-cell biotransformation of linolenic acid using the latter microorganisms can afford a mixture of (S)-(12Z)-10-hydroxy-octadecenoic acid (6), (S)-(9Z)-13-hydroxy-octadecenoic acid (8), and (10S,13S)-10,13-dihydroxy-octadecenoic acid (9). Furthermore, several probiotics aside from converting oleic and linoleic acid into acids 4 and 6, respectively, also produce the corresponding keto-derivatives 5 and 7 [31–34].

Overall, we can observe that to date, there has been no comprehensive study on the fatty acid hydratase activity of the probiotic microorganisms. By means of the present work, we try to fill this gap, focusing our study on the commercially available probiotics currently employed for human use. Accordingly, we undertook a comprehensive study intended to investigate the biocatalytic potential of the aforementioned strains in the transformation of oleic, linoleic, and linolenic acids. To this end, we evaluated their hydratase activity by measuring both yield and the regio- and stereoselectivity for each biotransformation experiment.

The obtained results, aside from extending those described by previous studies, give new insight on the in vivo fatty acid biotransformation as well as suggest the prospective utility of some probiotic strains for the preparative synthesis of enantioenriched HFAs and their derivatives.

2. Results and Discussion

In order to set up a comprehensive study on the fatty acid hydratase activity of the most relevant probiotics, we singled out thirteen strains. More specifically, we selected eight *Lactobacillus* species, namely *Lactobacillus rhamnosus* (ATCC 53103), *Lactobacillus plantarum* (299V), *Lactobacillus paracasei* (ATCC SD5275), *Lactobacillus bulgaricus* (GLB 44), *Lactobacillus reuteri* (DSM 17938), *Lactobacillus salivarius* (DSM 22775), *Lactobacillus gasseri* (SFB), *Lactobacillus acidophilus* (ATCC SD5212), and two *Bifidobacterium* strains, namely *Bifidobacterium animalis* subsp. *lactis* (DSM 15954) and *Bifidobacterium infantis* (35624). To complete our strain selection, we investigated the hydratase activity of two further bacteria, namely *Bacillus coagulans* (Colinox®) and *Streptococcus salivarius* (ATCC BAA 1024) as well as that of *Saccharomyces boulardii* (SB80®, CNCM I-3799), which is the only yeast widely used as a probiotic. All these microorganisms are commercially available because they are currently used to restore the gut and oral cavity flora and thus improve human health [43–55].

Subsequently, we defined the screening conditions. All the aforementioned strains were able to grow in an anaerobic environment, but with some remarkable differences. For example, the eight *Lactobacillus* species, *Streptococcus salivarius*, and *Bacillus coagulans* are facultative anaerobe or microaerophilic. In contrast, the two *Bifidobacterium* strains were anaerobic whereas *Saccharomyces boulardii* could grow both aerobically or anaerobically. Therefore, we decided to cultivate each one of the selected microorganisms in anaerobic conditions, at the physiological temperature of 37 °C.

The liquid media were selected according to the specific strain requirements. MRS medium and bifidobacterium medium were used to grow the *Lactobacillus* and *Bifidobacterium* species, respectively. *Bacillus coagulans*, *Streptococcus salivarius*, and *Saccharomyces boulardii* were grown using nutrient broth medium, tryptic soy broth medium, and universal medium for yeasts, respectively.

Since the hydration reaction is affected by the biotransformation conditions, we set up the following general screening protocol. Each microbial strain was grown in an anaerobic flask and the suitable fatty acid (3 g/L) was inoculated during the exponential phase of growth. After four days, the biotransformation mixtures were extracted. The metabolites were then derivatized and were analyzed by gas chromatography-mass spectrometry (GC-MS).

Based on the relative amount of the formed HFAs, we assigned the investigated microorganisms to one of the following three levels of hydratase activity. When the percentage of the produced HFAs, related to the sum of all fatty acid derivatives (including starting UFA) was lower than 1%, the strain is considered almost inactive. Otherwise, if the formed HFA percentage ranges between 5% and 1%, we considered the strain as able to perform the hydration reaction, even with low catalytic activity. Finally,

when the anaerobic flask biotransformation experiments indicated that the overall formation of the HFAs was higher than 5% of the sum of all fatty acid derivatives, we regarded these microbial strains as suitable biocatalysts for UFA hydration reactions. Therefore, the latter microorganisms were used in further biotransformation experiments performed in a larger scale by using a bioreactor.

Regarding the analysis of the enantiomeric composition of the HFAs, we measured these values according to the Rosazza procedure [56], which is based on the chemical transformation of the HFAs in the corresponding (S)-O-acetylmandelate derivatives of the methyl esters, followed by their $^1\text{H-NMR}$ analysis.

The above-described general protocol was applied to each of the biotransformation experiments. The hydratase activity were evaluated using three different substrates, namely oleic, linoleic, and linolenic acid. Each trial was performed in triplicate and the following results corresponded to the average of three data. Overall, the results obtained are collected in Table 1 and allow for a number of considerations.

Table 1. Results of the microbial biotransformation of oleic, linoleic, and linolenic acid by probiotic strains.

Microorganism	Hydratase Activity ¹		
	Starting Fatty Acid		
	Oleic	Linoleic	Linolenic
<i>Lactobacillus rhamnosus</i> ATCC 53103	(R)-4 (ee > 95%; yield 46%)	(S)-6 (ee > 95%; yield 47%)	(S)-10 (ee > 95%; yield 36%)
<i>Lactobacillus plantarum</i> 299V	(R)-4 (ee > 95%; yield 52%)	(S)-6 (ee > 95%; yield 46%)	(S)-10 (ee > 95%; yield 52%)
<i>Lactobacillus paracasei</i> ATCC SD5275	(R)-4 (ee > 95%; yield 45%)	(S)-6 (ee > 94%; yield 44%)	10 low activity ²
<i>Lactobacillus bulgaricus</i> GLB 44	(R)-4 (ee > 95%; yield 48%)	(S)-6 (ee > 95%; yield 41%)	10 low activity ²
<i>Lactobacillus reuteri</i> DSM 17938	(R)-4 (ee > 10%; yield 17%)	(S)-6 (ee > 78%; yield 5%)	no activity ³
<i>Lactobacillus salivarius</i> DSM 22775	(R)-4 (ee > 80%; yield 38%) 5 (6%)	(S)-6 (ee > 95%; yield 6%)	no activity ³
<i>Lactobacillus gasseri</i> SFB	(R)-4 (ee > 95%; yield 16%)	6 low activity ²	no activity ³
<i>Lactobacillus acidophilus</i> ATCC SD5212	(R)-4 (ee > 95%; yield 45%)	(S)-6 (ee > 95%; yield 10%) (S)-8 (ee > 95%; yield 41%) (10S,13S)-9 (yield 20%) ⁴	no activity ³
<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> DSM 15954	(R)-4 (ee > 90%; yield 51%)	6 low activity ²	no activity ³
<i>Bifidobacterium infantis</i> 35624	4 low activity ²	no activity ³	no activity ³
<i>Streptococcus salivarius</i> ATCC BAA 1024	(R)-4 (ee > 95%; yield 15%)	no activity ³	no activity ³
<i>Bacillus coagulans</i> Colinox [®]	no activity ³	no activity ³	no activity ³
<i>Saccharomyces boulardii</i> CNCM I-3799	no activity ³	no activity ³	no activity ³

¹ The biotransformation experiments lasted four days and were performed in a 5 L fermenter in anaerobic conditions at 37 °C and using a starting fatty acid concentration of 3 g/L (stirring 170 rpm, pH 6.2); yields were calculated on the basis of the weight of isolated hydroxyacids; ² According to preliminary experiments performed in an anaerobic flask, the hydroxy-acid derivatives were obtained in low yields (<5% by GC-MS analysis); ³ According to preliminary experiments performed in an anaerobic flask, the hydroxy-acid derivatives were not detected or their relative amounts were very low (<1% by GC-MS analysis); ⁴ The ee of compound 9 was not measured. Its NMR analysis indicates the presence of a single diastereoisomeric form. Assessing 2% of instrumental sensitivity, we can indicate a de > 96%.

First, we observed that the main part of the selected strains possessed hydratase activity, even if their stereoselectivity and biocatalytic efficiency changed dramatically depending on both the microbial strain and the fatty acid used. Among the evaluated probiotics, only *Lactobacillus rhamnosus* and *Lactobacillus plantarum* efficiently hydrated linolenic acid (**3**) to afford (S)-(12Z,15Z)-10-hydroxy-octadecadienoic (**10**) in high enantiomeric purity.

Lactobacillus paracasei and *Lactobacillus bulgaricus* were also able to transform linolenic acid into acid **10**, but they showed very low activity. On the contrary, each of the aforementioned four strains hydrated oleic acid (**1**) and linoleic acid (**2**) to give (R)-10-hydroxystearic acid (**4**), and (S)-(12Z)-10-hydroxy-octadecenoic acid (**6**), respectively, with good efficiency and in very high enantiomeric purity. It is worth noting that the described biotransformations proceeded with almost complete regiochemical control. In fact, only the 10-hydroxy derivatives were detected in the crude reaction mixtures and neither keto-derivatives nor other isomeric hydroxy-derivatives were formed. These observations also suggest that phylogenetically closely related species can display different biocatalytic activity and selectivity. In addition, the hydratase expression/activity of microorganisms belonging to the same species seem to be highly strain-dependent, as confirmed by our experiments using *Lactobacillus plantarum* (strain 299V). As demonstrated by our work, the UFA biotransformations performed using the latter strain afforded exclusively 10-hydroxy derivatives. In contrast, previous studies demonstrated that *Lactobacillus plantarum* (strain TMW1.460) [42] transformed linolenic acid in a mixture of (12Z)-10-hydroxy-octadecenoic acid (**6**) and (9Z)-13-hydroxy-octadecenoic acid (**8**), as result of the concurrent expression of both 10-linoleate hydratase and 13-linoleate hydratase.

Concerning the biotransformation experiments performed using *Lactobacillus reuteri*, *Lactobacillus salivarius*, and *Lactobacillus gasseri*, we observed a remarkable decrease of the hydratase activity, which was sometime characterized by low stereochemical control. The latter strains converted oleic acid into (R)-10-hydroxystearic acid (**4**) in modest yields and converted linoleic acid into (S)-(12Z)-10-hydroxy-octadecenoic acid (**6**) in low yields. In addition, *Lactobacillus reuteri* produces (R)-(**4**) and (S)-(**6**) with very low (10% ee) and modest (78% ee) enantiopurity, respectively. Similarly, *Lactobacillus salivarius* transforms oleic acid into (R)-(**4**) possessing modest enantiopurity (80% ee) and into 10-ketostearic acid (**5**). The latter ketone can result from either the hydration of oleic acid followed by the oxidation of the formed 10-hydroxystearic acid or through a multistep oxidation of oleic acid, not necessarily involving the transformation of 10-hydroxystearic acid (Figure 3).

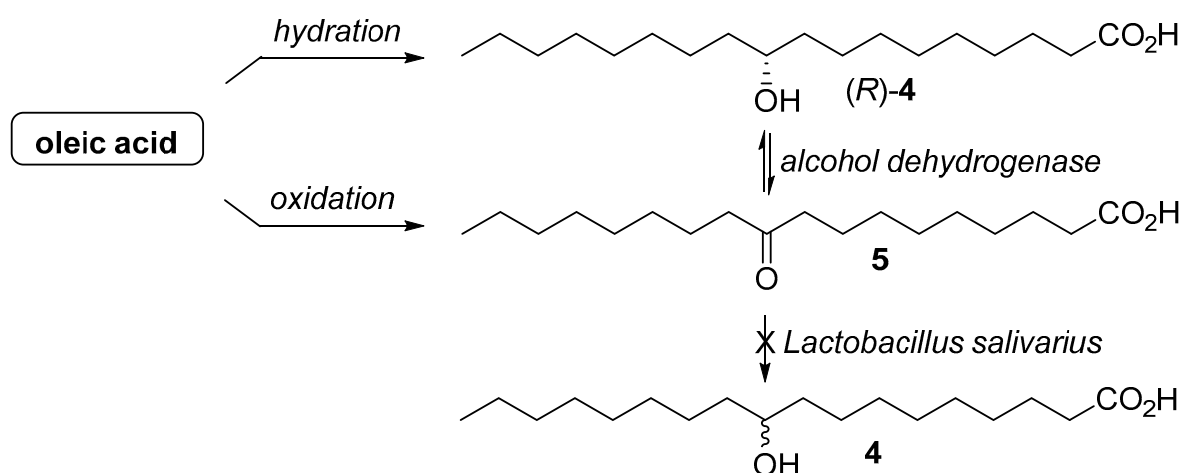


Figure 3. Two possible biosynthetic pathways involved in the *Lactobacillus salivarius* mediated transformation of oleic acid into 10-HSA (**4**) and 10-KSA (**5**).

The two step process, which involves the action of alcohol dehydrogenases (ADs), can justify the presence of 10-hydroxystearic acid in low enantiomeric purity, as the result of the alcohol/ketone equilibration catalyzed by ADs possessing modest enantioselectivity. It is worth noting that other

studies have reported similar observations, but the postulated mechanism should involve the presence of secondary alcohol dehydrogenases, which are essential for the formation of the 10-ketostearic acid (5) starting from 10-HSA. In order to clarify this point, we set up a new experiment in which a sample of 10-KSA (3 g/L) was added to a fermenting culture of *Lactobacillus salivarius*. In these conditions, we did not observe the formation of 10-HSA, thus indicating that oleic acid was oxidized to 10-KSA through a more complex pathway, possibly involving different enzymatic activities.

Remarkably, the probiotic strain of *Lactobacillus acidophilus* (ATCC SD5212) displayed very relevant hydratase activity. More specifically, we observed the action of both 10- and 13-hydratase activities. As described for other *Lactobacillus* strains, the biotransformation of oleic acid afforded (*R*)-10-hydroxystearic acid (4) in good yield and with high enantioselectivity. In contrast, linoleic acid was transformed into a mixture of (12*Z*)-10-hydroxy-octadecenoic acid (6), (9*Z*)-13-hydroxy-octadecenoic acid (8), and 10,13-dihydroxy-octadecenoic acid (9a) in high overall yield. Both absolute configuration and enantiomeric purity of compounds 6 and 8 were determined by NMR analysis of their corresponding (*S*)-O-acetylmandelate derivatives of the methyl esters, according to previously reported studies [12,39]. These means confirmed that the investigated microorganism holds hydratase activity similar to that reported for other *Lactobacillus acidophilus* strains, affording (*S*)-(12*Z*)-10-hydroxy-octadecenoic acid (6) and (*S*)-(9*Z*)-13-hydroxy-octadecenoic acid (8) in high enantiomeric purity. The simultaneous activity of two different hydratases can justify the formation of the diol 9a (Figure 4), which can be generated through a two-step hydration pathway.

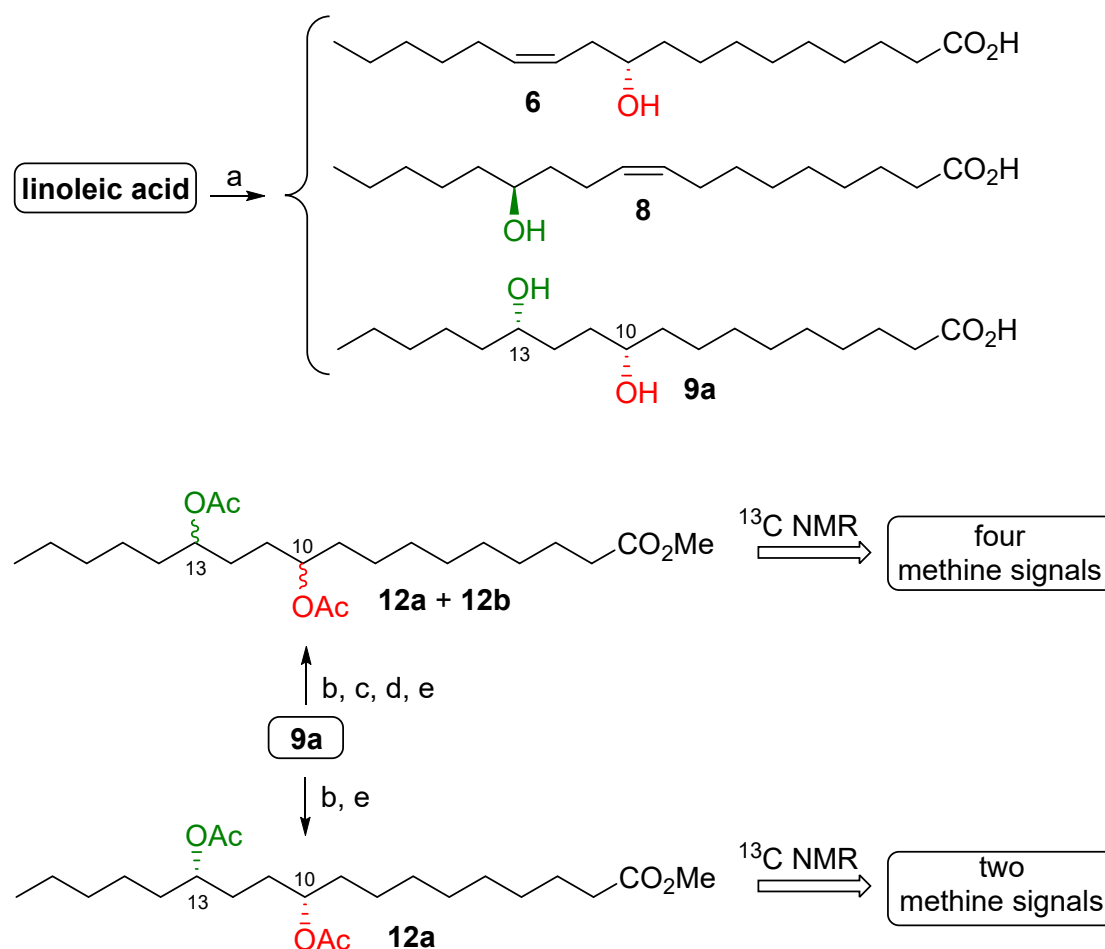


Figure 4. Biotransformation of linoleic acid (2) by *Lactobacillus acidophilus* (ATCC SD5212) and demonstration that diol 9a is produced as a single diastereoisomeric form. Reagents and conditions: (a) Fermentation with *Lactobacillus acidophilus* (ATCC SD5212); (b) CH₂N₂, Et₂O, 0 °C; (c) DMSO, ClCOOCl, Et₃N, CH₂Cl₂, −70 °C; (d) NaBH₄, MeOH, 0 °C then HCl aq. (3% w/v); (e) Ac₂O, Py, DMAP cat., rt.

Concerning the stereochemistry of diol **9a**, we tentatively assigned the 10*S*,13*S* absolute configuration to 10,13-dihydroxy-octadecenoic acid as the result of the 10- and 13-linoleate hydratase. It is reasonable to suppose that the enzymes responsible for the formation of compounds **6** and **8** are also involved in the biosynthesis of diol, thus affording the 10*S*,13*S* enantiomer. Despite this, the assumption that the stereoselectivity of the hydration of the intermediate HFAs is the same as those measured for the first hydration steps of linolenic acid, should not be taken for granted. In order to investigate this point further, we measured the diastereoisomeric purity of diol **9a** by ¹H- and ¹³C-NMR analysis of **9a** and of its derivatives (diacetate methyl ester **12a**).

We observed that diol **9a** seems to be produced as a single diastereoisomeric form. The strict confirmation of this data was acquired through the analysis of the equimolar mixture of the diastereoisomeric derivatives **12a** and **12b**, obtained by chemical synthesis (Figure 4). Accordingly, Swern oxidation of the methyl ester of diol **9a** furnished the corresponding diketo derivative that was not isolated and was reduced using NaBH₄ in methanol. The latter reaction gave the desired diastereoisomeric mixture of racemic diols. The ¹³C-NMR analysis of the latter mixture (derivatized as diacetate) showed the presence of four methine carbon signals (at 74.09, 74.06, 73.90, and 73.87 ppm), whereas the same analysis performed on the compound **12a**, obtained from diol **9a**, indicated the presence of only two methine carbon signals (at 74.09 and 74.06 ppm). Seen together, these data suggest that 10- and 13-linoleate hydratases produced by *Lactobacillus acidophilus* work independently and their catalytic activity is not affected by the presence of a preexisting hydroxy group thus affording diol **9a** in a single diastereoisomeric form, possessing the 10*S*,13*S* absolute configuration. Interestingly, the biocatalytic activity of the latter *Lactobacillus acidophilus* strain toward linolenic acid was negligible and the HFA derivatives **10** and **11** were not detected in the crude biotransformation mixture. This experimental results do not agree with a similar biotransformation process [30] that transforms linolenic acid into compound **11**, making use of recombinant *Escherichia coli* expressing linoleate hydratase from a different *Lactobacillus acidophilus* strain.

A completely different hydratase activity was observed for the two *Bifidobacterium* species investigated. Both probiotics did not significantly transform linoleic and linolenic acids, but were able to hydrate oleic acid, although with very different transformation yields. *Bifidobacterium animalis* subsp. *lactis* transformed oleic acid into (R)-10-HSA in good yield (51%) and high enantiomeric purity (90% ee). In contrast, *Bifidobacterium infantis* afforded only a minor amount of 10-HSA, whose absolute configuration was not determined.

Next, we studied three strains that do not belong to the *Lactobacillus* and *Bifidobacterium* genus, namely *Streptococcus salivarius*, *Bacillus coagulans*, and *Saccharomyces boulardii*. *Streptococcus salivarius* did not show hydratase activity toward linoleic and linolenic acids, whereas it transformed oleic acid into 10-HSA in modest yield (15%) and in very high enantiomeric purity (95% ee). Moreover, *Bacillus coagulans* and *Saccharomyces boulardii* were not able to hydrate any of the investigated fatty acids.

The results obtained with the latter microorganism seem to confirm the validity of our previous study on the baker's yeast-mediated hydration of oleic acid [57]. Through the aforementioned work, we established that *Saccharomyces cerevisiae* does not possess hydratase activity, whilst the bacterial contaminants of commercial baker's yeast are effectively responsible for the stereoselective formation of oleic acid into (R)-10-hydroxystearic acid. Since *Saccharomyces boulardii* is morphologically, physiologically, and genetically very close to *Saccharomyces cerevisiae*, we regard the experimental data obtained by the present work as further confirmation of our previous findings.

It is worth noting that the *Lactobacillus* species are microaerophilic, the two *Bifidobacterium* strains are strict anaerobic bacteria, whereas *Streptococcus salivarius*, *Bacillus coagulans*, and *Saccharomyces boulardii* can grow both aerobically or anaerobically. In order to compare homogeneous results, we studied the hydration reaction using the same experimental conditions for each microorganism and the data reported in Table 1 correspond to anaerobic biotransformation. Moreover, for the sake of completeness, we evaluated the hydration activity of the three latter strains in both aerobic and anaerobic conditions using oleic acid as the substrate (Table 2).

Table 2. Results of the microbial biotransformation of oleic acid by three anaerobic facultative probiotic strains.

Microorganism	Hydratase Activity Versus Oleic Acid ¹		
	Experimental Conditions		
	Aerobic Flask ¹	Anaerobic Flask ¹	Bioreactor (Anaerobic) ¹
<i>Streptococcus salivarius</i> ATCC BAA 1024	4 (yield 1%)	4 (yield 5%)	(R)-4 (ee 95%; yield 15%)
<i>Bacillus coagulans</i> Colinox [®]	no activity ²	no activity ²	no activity ²
<i>Saccharomyces boulardii</i> CNCM I-3799	no activity ²	no activity ²	no activity ²

¹ The biotransformation experiments, performed in flask, were kept at 37 °C, 130 rpm for 4 days and using a starting oleic acid concentration of 3 g/L; the bioreactor settings are described above (Table 1); yields were calculated on the basis of either GC-MS analysis (flask fermentation) or on the weight of the isolated hydroxy acids (bioreactor fermentation); ² The hydroxy-acid derivatives were not detected by GC-MS analysis or their relative amounts were very low (<1%).

We observed that *Bacillus coagulans* and *Saccharomyces boulardii* were completely inactive, independent of the experimental conditions. Otherwise, the 10-HSA produced by *Streptococcus salivarius* in an aerobic flask (1% yield) was about five times lower than that obtained performing the hydration reaction using an anaerobic flask (5% yield). Moreover, the yield of the same biotransformation improved further by using a bioreactor, setting anaerobic conditions, and carefully controlling the pH of the medium. Through these means, we found an increase in the 10-HSA yields of up to 15%. Seen together, these results further confirm that the experimental conditions strongly affected the biotransformation yields.

3. Materials and Methods

3.1. Materials and General Methods

All air and moisture sensitive reactions were carried out using dry solvents and under a static atmosphere of nitrogen. All solvents and reagents were of commercial quality and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Oleic acid (94%, lot. MKBZ2615V), linoleic acid (99%, lot. SLBT2627), linolenic acid (68%, lot. 310689/1), casein peptone, peptone from soybean, yeast extract, meat extract, malt extract, tryptic soy broth, sodium thioglycolate, sodium formaldehyde sulfoxylate, resazurin sodium salt, L-cysteine, and glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Linolenic acid (85% purity, lot. 81003) was purchased from Nissan—Nippon Oil and Fats Co. (Tokyo, Japan), Ltd. (S)-O-acetyl mandelic acid was prepared starting from (S)-mandelic acid and using acetic anhydride, pyridine and cat. DMAP, as described previously [58].

Reference standard samples of (R)-10-hydroxystearic acid, (S)-(12Z)-10-hydroxy-octadecenoic acid, and (S)-(12Z,15Z)-10-hydroxy-octadecadienoic acid (all with ee > 95%) were prepared by the *Lactobacillus rhamnosus* mediated hydration of oleic, linoleic, and linolenic acids, respectively [12].

(S)-(9Z)-13-hydroxy-octadecenoic acid (ee > 95%) was prepared by the *Lactobacillus acidophilus* mediated hydration of linoleic acid.

A reference standard sample of 10-ketostearic acid was prepared by the oxidation of (R)-10-hydroxystearic acid [57].

A reference standard sample of 10-(R)-hydroxystearic acid, showing 21% ee, was prepared by the baker's yeast-mediated hydration of oleic acid [57]. Reference standard samples of the methyl esters of the racemic (12Z)-10-hydroxy-octadecenoic acid, (9Z)-13-hydroxy-octadecenoic acid and (12Z,15Z)-10-hydroxy-octadecadienoic acid were prepared starting from the corresponding (S) enantiomeric forms. The racemization process was based on the following two step chemical transformation.

A sample of the enantio-enriched HFA (300 mg, 1 mmol) was treated with an excess of an ethereal solution of freshly prepared diazomethane. As soon as the evolution of nitrogen ceased, the solvent was eliminated and the residue was dissolved in CH_2Cl_2 (2 mL). Parallel to this reaction, a solution of dry DMSO (0.5 mL, 7 mmol) in CH_2Cl_2 (3 mL) was added dropwise to a stirred solution of oxalyl chloride (0.3 mL, 3.5 mmol) in CH_2Cl_2 (7 mL) at -70°C . After ten minutes, the solution of the fatty acid methyl ester described above was added dropwise. After a further 15 min, dry Et_3N (2 mL, 14.3 mmol) was added and the resulting mixture was allowed to warm to room temperature. The reaction was then poured into ice-cooled water and extracted twice with CH_2Cl_2 (50 mL \times 2). The combined organic phases were washed with brine and concentrated under reduced pressure. The residue was dissolved in methanol (30 mL) and was treated at 0°C with NaBH_4 (100 mg, 2.6 mmol) under stirring. After complete reduction of the ketone (TLC analysis), the reaction was quenched by the addition of diluted HCl aq. (3% *w/w*, 40 mL), followed by extraction with CH_2Cl_2 (50 mL \times 2). The combined organic phases were washed with brine and concentrated under reduced pressure. The residue was purified by chromatography using *n*-hexane/ AcOEt (9:1–7:3) as the eluent to afford the racemic hydroxy acid derivatives (180–230 mg, 60–77% yield).

The above described procedure was employed for the synthesis of racemic 10,13-dihydroxy-octadecenoic acid methyl ester (the methyl ester of an equimolar mixture of compounds **9a** and **9b**). The only modification concerns the amount of the starting hydroxy acid. We used 160 mg (0.5 mmol) of diol **9a** instead of 300 mg of the HFA (1 mmol).

3.2. Analytical Methods and Characterization of the Products Deriving from the Biotransformation Experiments

3.2.1. Instruments and Analytic Condition

Nuclear magnetic resonance spectroscopy (NMR): ^1H - and ^{13}C -NMR Spectra and DEPT experiments: CDCl_3 solutions at room temperature (r.t.) using a Bruker-AC-400 spectrometer (Billerica, MA, USA) at 400, 100, and 100 MHz, respectively; the ^{13}C spectra are proton decoupled; chemical shifts in ppm relative to internal SiMe_4 ($= 0$ ppm).

TLC: Merck silica gel 60 F_{254} plates (Merck Millipore, Milan, Italy).

Column chromatography: silica gel.

Melting points were measured on a Reichert apparatus (Reichert, Vienna, Austria), equipped with a Reichert microscope, and are uncorrected.

Optical rotations were measured on a Jasco-DIP-181 digital polarimeter (Tokyo, Japan).

Mass spectra were recorded on a Bruker ESQUIRE 3000 PLUS spectrometer (ESI detector) (Billerica, MA, USA) or by GC-MS analyses.

GC-MS analyses: A HP-6890 gas chromatograph equipped with a 5973 mass detector using a HP-5MS column (30 m \times 0.25 mm, 0.25 μm film thickness; Hewlett Packard, Palo Alto, CA, USA) was used with the following temperature program: 120° (3 min)— $12^\circ/\text{min}$ — 195° (10 min)— $12^\circ/\text{min}$ — 300° (10 min); carrier gas: He; constant flow 1 mL/min; split ratio: 1/30; t_R given in minutes.

3.2.2. GC-MS Analyses

The biotransformations of oleic acid, linoleic acid, and linolenic acid to give 10-hydroxystearic acid (**4**), 10-ketostearic acid (**5**), (12*Z*)-10-hydroxy-octadecenoic acid (**6**), (9*Z*)-13-hydroxy-octadecenoic acid (**8**), 10,13-dihydroxy-octadecenoic acid (**9**), and (12*Z*,15*Z*)-10-hydroxy-octadecadienoic acid (**10**) were monitored by means of GC-MS analysis. To this end, the biotransformation mixture was acidified at pH 4 and filtered on celite. The aqueous phase was then extracted three times with ethyl acetate and the combined organic layer was washed with brine and dried on Na_2SO_4 . The solvent was then removed under reduced pressure and the residue was treated at 0°C with an excess of an ethereal solution of freshly-prepared diazomethane. As soon as the evolution of nitrogen ceased, the solvent was eliminated and the residue was treated at RT with a 1:1 mixture of pyridine/acetic anhydride

(4 mL for about 100 mg of residue) and DMAP (10 mg). After five hours, the excess of reagents were removed in vacuo and the residue was analyzed by GC-MS.

Oleic acid methyl ester: t_R 18.95

GC-MS (EI): m/z (%) = 296 [M^+] (7), 264 (49), 235 (6), 222 (30), 180 (19), 166 (10), 152 (12), 137 (17), 123 (26), 110 (32), 97 (62), 83 (68), 69 (79), 55 (100).

Linoleic acid methyl ester: t_R 18.52

GC-MS (EI): m/z (%) = 294 [M^+] (18), 263 (15), 234 (1), 220 (4), 178 (6), 164 (10), 150 (16), 135 (15), 123 (18), 109 (36), 95 (70), 81 (93), 67 (100), 55 (56).

Linolenic acid methyl ester: t_R 18.79

GC-MS (EI): m/z (%) = 292 [M^+] (7), 261 (4), 249 (2), 236 (5), 191 (3), 173 (5), 149 (13), 135 (15), 121 (20), 108 (34), 95 (56), 79 (100), 67 (66), 55 (43).

Methyl 10-Ketostearate: t_R 23.37

GC-MS (EI): m/z (%) = 312 [M^+] (2), 281 (23), 239 (5), 227 (5), 214 (52), 199 (40), 182 (11), 156 (100), 141 (67), 125 (86), 97 (60), 81 (31), 71 (90), 55 (89)

Methyl 10-acetoxystearate: t_R 24.47

GC-MS (EI): m/z (%) = 313 [M^+ -MeCO] (6), 296 [M^+ -AcOH] (3), 281 (17), 264 (31), 243 (11), 222 (9), 201 (100), 169 (64), 157 (16), 125 (21), 97 (18), 83 (19), 69 (21), 55 (27).

Methyl (12Z)-10-acetoxy-octadecenoate: t_R 24.28

GC-MS (EI): m/z (%) 311 [M^+ -MeCO] (<1), 294 [M^+ -AcOH] (39), 279 (1), 263 (24), 220 (7), 201 (46), 169 (100), 150 (13), 136 (9), 123 (15), 109 (21), 95 (37), 81 (53), 67 (46), 55 (32).

Methyl (9Z)-13-acetoxy-octadecenoate: t_R 24.05

GC-MS (EI): m/z (%) 311 [M^+ -MeCO] (<1), 294 [M^+ -AcOH] (40), 279 (1), 263 (29), 241 (3), 220 (9), 210 (10), 196 (12), 178 (22), 164 (28), 150 (24), 136 (26), 123 (27), 109 (41), 95 (78), 81 (100), 67 (95), 55 (79).

Methyl (12Z,15Z)-10-acetoxy-octadecadienoate: t_R 24.33

GC-MS (EI): m/z (%) 292 [M^+ -AcOH] (76), 277 (1), 261 (20), 201 (33), 169 (100), 149 (19), 135 (28), 121 (41), 108 (42), 93 (57), 79 (87), 55 (39).

Methyl (10S,13S)-diacetoxy-stearate (12a): t_R 26.61

GC-MS (EI): m/z (%) 383 [M^+ -OMe] (2), 355 (1), 336 (1), 323 (2), 311 (11), 294 (58), 279 (6), 263 (42), 241 (70), 214 (18), 201 (41), 169 (48), 141 (100), 123 (47), 109 (25), 95 (50), 81 (78), 67 (58), 55 (69).

An equimolar mixture of compounds **12a** and **12b** gave a single peak by GC-MS analysis, thus indicating that the two diastereoisomers had the same retention time.

3.2.3. Determination of the Absolute Configuration and of the Optical Purity of the HFAs

The enantiomeric composition of the isolated 10-hydroxystearic acid, (12Z)-10-hydroxy-octadecenoic acid, (9Z)-13-hydroxy-octadecenoic acid, and (12Z,15Z)-10-hydroxy-octadecadienoic acid samples, obtained from the biotransformation experiments, was determined by $^1\text{H-NMR}$ analysis, according to the Rosazza procedure [56]. Hence, each of the hydroxy acid samples (100 mg, 0.33 mmol) was treated with an excess of an ethereal solution of freshly-prepared diazomethane. As soon as the evolution of nitrogen ceased, the solvent was eliminated and the resulting methyl ester was dissolved in dry CH_2Cl_2 (5 mL) treated with (S)-O-acetylmandelic acid (130 mg, 0.67 mmol), DCC (140 mg, 0.68 mmol), and DMAP (10 mg), with stirring at RT for 6 h. The reaction was then quenched by the addition of water and diethyl ether (60 mL). The formed dicyclohexylurea was removed by filtration on celite and the organic phase was washed with aq. NaHCO_3 , brine, and dried on Na_2SO_4 . The solvent was then removed under reduced pressure and the residue was roughly purified by chromatography, collecting every fraction containing the fatty acid mandelates. As previously described [12], the $^1\text{H-NMR}$ analysis of the obtained (S)-O-acetylmandelates allowed the determination of the absolute configuration of the starting hydroxy acids as well as the measurement of their optical purity.

Concerning (9Z)-13-hydroxy-octadecenoic acid samples, their absolute configuration was assigned by comparing the $^1\text{H-NMR}$ analysis of the corresponding (S)-O-acetylmandelates with that reported for the same derivative obtained, starting from (S)-(9Z)-13-hydroxy-octadecenoic acid [39].

3.3. Microorganisms and Biotransformation Experiments

3.3.1. Microorganisms and Media

Lactobacillus rhamnosus (ATCC 53103, trade name Kaleidon 60) was purchased from Malesci Spa (Bagno a Ripoli, Italy).

Lactobacillus bulgaricus (GLB 44) was purchased from GENESIS GLB44 PROBIOTIC – GENESIS LABORATORIES Ltd. (Sofia, Bulgaria).

Lactobacillus acidophilus (La-14 = ATCC SD5212, trade name GSE AcidophiPlus) was purchased from Prodeco Pharma S.r.l (Castelfranco Veneto, Italy).

Lactobacillus plantarum (299V, trade name Smebiocta LP299V) was purchased from Ipsen Pharma (Boulogne-Billancourt, France).

Lactobacillus gasseri (SFB) was purchased from SFB Laboratoires (Saint-Pierre du Perray, France).

Lactobacillus paracasei (Lpc 37 = ATCC SD5275, trade name Flortec) was purchased from Procemsa S.p.A. (Nichelino, Italy).

Lactobacillus reuteri (DSM 17938, trade name Reuflor) was purchased from Italchimici S.p.A. (Italy).

Lactobacillus salivarius (LS01 = DSM 22775, trade name FlorAtopic) was purchased from Probiotal S.p.A. (Novara, Italy).

Bifidobacterium animalis subsp. *lactis* (BB-12 = DSM 15954, trade name Bifido Lactis Infant) was purchased from SOFAR S.p.A. (Trezzano Rosa, Italy).

Bifidobacterium infantis (35624, trade name Alflorex) was purchased from BIOCODEX (Gentilly Cedex, France).

Bacillus coagulans (Colinox[®]) was purchased from DMG Italia S.r.l. (Pomezia, Italy).

Streptococcus salivarius (BLIS K12 = ATCC BAA 1024, trade name Bactoblis) was purchased from Omeopiacenza S.r.l. (Pontenure, Italy).

Saccharomyces boulardii (probiotic strain SB80[®], CNCM I-3799, trade name Codex) was purchased from AR Fitofarma S.r.l. (Assago, Italy).

The biotransformation experiments were performed using five different media, namely the MRS Medium (MRS), the Bifidobacterium Medium (BM), the Nutrient Broth Medium (NB), the Tryptic Soy Broth Medium (TSB), and the universal Medium for Yeasts (YM), depending on the microorganism used.

MRS composition: casein peptone (10 g/L), meat extract (10 g/L), yeast extract (5 g/L), glucose (20 g/L), Tween 80 (1 mL/L), K₂HPO₄ (2 g/L), NaOAc (5 g/L), ammonium citrate dibasic (2 g/L), MgSO₄·7H₂O (0.2 g/L) MnSO₄·H₂O (50 mg/L), L-cysteine 0.1% (w/w), sodium thioglycolate (2 g/L).

BM composition: casein peptone (10 g/L), peptone from soybean (5 g/L), yeast extract (5 g/L), meat extract (5 g/L), glucose (10 g/L), NaCl (5 g/L), K₂HPO₄ (2 g/L), Tween 80 (1 mL/L), MgSO₄·7H₂O (0.2 g/L) MnSO₄·H₂O (50 mg/L), L-cysteine 0.1% (w/w), sodium thioglycolate (2 g/L), sodium formaldehyde sulfoxylate (1 g/L), salt solution [CaCl₂·2H₂O (0.25 g/L), MgSO₄·7H₂O (0.5 g/L), KH₂PO₄ (1 g/L), K₂HPO₄ (1 g/L), NaHCO₃ (10 g/L), NaCl (2 g/L)] 40 mL/L.

NB composition: casein peptone (3 g/L), peptone from soybean (3 g/L), meat extract (3 g/L), MnSO₄·H₂O (10 mg/L).

TSB composition: tryptic soy broth (30 g/L), yeast extract (3 g/L)

YM composition: yeast extract (3 g/L), malt extract (3 g/L), peptone from soybeans (5 g/L), glucose (10 g/L).

MRS was used for all *Lactobacillus* species.

BM was used for all *Bifidobacterium* species.

NB was used for *Bacillus coagulans*.

TSB was used for *Streptococcus salivarius*.

YM was used for *Saccharomyces boulardii*.

All the biotransformations were carried out in triplicate and the presented results are the media of three experimental data.

The experimental conditions used for the biotransformations depended on the use of anaerobic/aerobic conditions and on the decision to perform the experiments on a flask scale or using a bioreactor.

All the preparative biotransformation experiments were performed using a 5 L fermenter (Biostat A BB-8822000, Sartorius-Stedim (Göttingen, Germany) in anaerobic conditions.

3.3.2. General Procedure for the Biotransformation Experiments Using Anaerobic Flasks

The anaerobic flasks were prepared loading 40 mL of the suitable medium (MRS, BM, NB, TSB, and YM media) in 100 mL conical vacuum flasks followed by the addition of cysteine (for NB, TSB, and YM media; 40 mg), and resazurine sodium salt (1 mg) or methylene blue (for BM; 1 mg). The flasks were flushed with nitrogen until the complete removal of the oxygen content, then were sealed with silicone rubber septa and sterilized (121 °C, 15 min). Each flask was inoculated via syringe with the suitable lyophilized bacteria strain (about 4×10^9 CFU, suspended in 2 mL of sterilized skimmed milk), or with *Saccharomyces boulardii* (about 0.4 g of wet cells obtained by centrifugation for 5 min at 3220 g of an active culture, suspended in 2 mL of sterilized saline solution). Then, the flasks were incubated at 37 °C and at 130 rpm.

A solution of the fatty acid (120 mg) in ethanol (0.15 mL) and 2 mL of a sterilized solution of glucose (300 g/L) in water were added to each flask after 3.5 and 8 h since the inoculum, respectively. After four days, the reaction mixtures were acidified at pH 4 by addition of diluted HCl and then filtered on celite. The aqueous phases were then extracted three times with ethyl acetate and the combined organic layers were washed with brine, dried on Na₂SO₄, and the solvent was removed under reduced pressure. The crude biotransformation mixtures were derivatized and analyzed by GC-MS as described above (Section 3.2.2).

3.3.3. General Procedure for the Biotransformation Experiments Using Aerobic Flasks

The aerobic flasks were prepared loading 40 mL of the suitable medium (NB, TSB, and YM media) in 100 mL conical vacuum flasks. The flasks were sealed with a cellulose plug and sterilized (121 °C, 15 min.). Each flask was inoculated with the suitable lyophilized bacteria strain (about 4×10^9 CFU, suspended in 2 mL of sterilized skimmed milk) or with *Saccharomyces boulardii* (about 0.4 g of wet cells obtained by centrifugation for 5 min at 3220 g of an active culture, suspended in 2 mL of sterilized saline solution). Then, the flasks were incubated at 37 °C and at 130 rpm.

A solution of the fatty acid (120 mg) in ethanol (0.15 mL) and 2 mL of a sterilized solution of glucose (300 g/L) in water were added to each flask after 3.5 and 8 h since the inoculum, respectively. After four days, the reaction mixtures were acidified at pH 4 by the addition of diluted HCl and then were filtered on celite. The aqueous phases were then extracted three times with ethyl acetate and the combined organic layers were washed with brine, dried on Na₂SO₄, and the solvent was removed under reduced pressure. The crude biotransformation mixtures were derivatized and analyzed by GC-MS as described above (Section 3.2.2).

3.3.4. General Procedure for Preparative Biotransformations

Two anaerobic flasks, containing 40 mL of the MRS or BM medium, were prepared as described above, were inoculated with the suitable *Lactobacillus*, *Bifidobacterium*, or *Streptococcus* strain (about 4×10^9 CFU for each flask) and then were incubated at 37 °C and 130 rpm for 12 h. The cultures were centrifuged at $3220 \times g$ for 3 min (4 °C), the supernatant removed, and the cells were resuspended in 5 mL of sterilized skimmed milk. The obtained suspension was added to a sterilized fermenter vessel containing the suitable nitrogen flushed medium (1 L). The temperature, the stirring speed, and the pH were set to 37 °C, 170 rpm, and 6.2, respectively. The pH was controlled by the dropwise addition of sterilized aqueous solutions (10% w/w in water) of either acetic acid or ammonia.

After some hours (2–8 h, depending of the strain used) the fermentation showed an exponential phase of growth, as indicated by starting with the continuous addition of the base, necessary to

neutralize the lactic acid produced by the glucose bacterial catabolism. At this point, a solution of the suitable fatty acid (3 g) in ethanol (5 mL) was added dropwise. As soon as the lactic acid production decreased (about 24 h), 50 mL of a sterilized solution of glucose (300 g/L) in water was further added. The fermentation was stopped four days since the inoculum of the bacteria by means of acidification of the reaction mixture with diluted HCl (pH 4), followed by the filtration of the biomass through a celite pad. The aqueous phase was then extracted three times with ethyl acetate and the combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. The residue was purified by chromatography using *n*-hexane/AcOEt (9:1–1:2) as the eluent to afford unreacted fatty acid (first eluted fractions), followed by keto or hydroxy acid derivatives.

The compounds derived from preparative biotransformation reactions were characterized by GC-MS, ESI-MS, and NMR analyses, whereas their enantiomeric purity was determined as described above (Section 3.2.3).

The NMR data and the optical rotation values measured for HFAs **4**, **6**, and **10** were superimposable to those described in our previous work [12]. The NMR data recorded for 10-ketostearic acid **5** were in accordance with those recorded for the synthetic acid [57].

The compounds **8**, **9a**, and **12a** were fully characterized and showed the following analytical data: (S)-(9Z)-13-hydroxy-octadecenoic acid (**8**): $[\alpha]_D^{20} = +0.2$ (c 2.4, CHCl_3).

^1H NMR (400 MHz, CDCl_3) δ 5.75 (br s, 1H), 5.43–5.32 (m, 2H), 3.68–3.57 (m, 1H), 2.34 (t, $J = 7.4$ Hz, 2H), 2.24–1.97 (m, 4H), 1.70–1.58 (m, 2H), 1.58–1.21 (m, 18H), 0.89 (t, $J = 6.8$ Hz, 3H).

^{13}C NMR (100 MHz, CDCl_3) δ 179.2 (C), 130.4 (CH), 129.3 (CH), 71.9 (CH), 37.3 (CH_2), 37.2 (CH_2), 33.9 (CH_2), 31.9 (CH_2), 29.4 (CH_2), 28.9 (CH_2), 28.9 (CH_2), 28.9 (CH_2), 27.1 (CH_2), 25.3 (CH_2), 24.6 (CH_2), 23.5 (CH_2), 22.6 (CH_2), 14.0 (Me).

MS (ESI): 321.2 ($\text{M} + \text{Na}^+$); 297.0 ($\text{M}-1$, negative ions)

(10S,13S)-Dihydroxystearic acid (**9a**): M.p: 97–98 °C; $[\alpha]_D^{20} = -1.0$ (c 2.5, MeOH).

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 11.30 (br s, 1H), 4.16 (br s, 2H), 3.41–3.25 (br s, 2H), 2.16 (t, $J = 7.4$ Hz, 2H), 1.56–1.11 (m, 26H), 0.85 (t, $J = 6.8$ Hz, 3H).

^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 174.3 (C), 69.9 (CH), 37.0 (CH_2), 37.0 (CH_2), 33.5 (CH_2), 33.3 (CH_2), 31.4 (CH_2), 29.1 (CH_2), 28.9 (CH_2), 28.6 (CH_2), 28.5 (CH_2), 25.1 (CH_2), 24.8 (CH_2), 24.4 (CH_2), 22.0 (CH_2), 13.8 (Me).

MS (ESI): 339.2 ($\text{M} + \text{Na}^+$); 315.0 ($\text{M}-1$, negative ions).

(10S,13S)-Methyl diacetoxystearic acid (**12a**): $[\alpha]_D^{20} = +0.7$ (c 3.3, CHCl_3). ^1H NMR (400 MHz, CDCl_3) δ 4.89–4.77 (m, 2H), 3.66 (s, 3H), 2.30 (t, $J = 7.5$ Hz, 2H), 2.04 (s, 6H), 1.69–1.41 (m, 10H), 1.38–1.18 (m, 16H), 0.88 (t, $J = 6.8$ Hz, 3H).

^{13}C NMR (100 MHz, CDCl_3) δ 174.2 (C), 170.8 (C), 74.1 (CH), 74.1 (CH), 51.4 (Me), 34.0 (CH_2), 34.0 (CH_2), 34.0 (CH_2), 31.6 (CH_2), 29.8 (CH_2), 29.4 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 29.0 (CH_2), 25.2 (CH_2), 24.9 (CH_2), 22.5 (CH_2), 21.2 (Me), 13.9 (Me).

The ^1H NMR of the equimolar mixture of compounds **12a** and **12b** was superimposable to that described for compound **12a**.

The ^{13}C NMR spectra of the same mixture allowed for the unambiguous identification of four methine signals at 74.09, 74.06, 73.90, and 73.87 ppm, whereas compound **12a** showed only two methine signals (at 74.09 and 74.06 ppm).

4. Conclusions

The present work demonstrates that many probiotic bacteria, currently used to improve human health, are able to catalyze the hydration of oleic, linoleic, and linolenic acids. The latter unsaturated fatty acids are components, as triglycerides, of the most common vegetable oils employed in food. Therefore, the biocatalytic competencies of these microorganisms have a dual significance. First, the determination of the metabolites that can be produced by a given strain, which is usually present in the gut flora, represent a finding of scientific relevance, especially in the medical field.

A second, very important aspect, concerns the biotechnological potential of these microorganisms. We demonstrated that the investigated probiotics usually catalyze the hydration reaction of UFAs with very high regio- and stereoselectivity. Our biotransformation experiments almost exclusively afforded 10-HFA derivatives with the single exception of *Lactobacillus acidophilus* ATCC SD5212, which converted linoleic acid in a mixture of 13-HFAs and 10-HFAs derivatives. The most accepted substrate was oleic acid, followed by linoleic and linolenic acids, with this order of preference. The latter three acids were transformed into (*R*)-10-hydroxystearic acid, (*S*)-(12*Z*)-10-hydroxy-octadecenoic, and (*S*)-(12*Z*,15*Z*)-10-hydroxy-octadecadienoic acids, respectively, usually with very high enantiomeric purity (ee > 95%). Concerning biotransformation at relatively high substrate concentration (3 g/L), we observed that yields did not exceed 52%, even for the most efficient microorganisms, pointing to a possible inhibition of enzymatic activity due to the product itself. Among the thirteen investigated strains, *Lactobacillus rhamnosus* ATCC 53103 and *Lactobacillus plantarum* 299 V proved to be the most versatile, being able to efficiently and selectively hydrate all three investigated fatty acids. Overall, this study furnishes the scientific basis for the exploitation of probiotic bacteria for the biotechnological production of HFAs.

Author Contributions: S.S. and D.D.S. conceived this study; S.S., D.D.S., A.C., and M.V. equally contributed to the design and performed the experiments as well as analyzed the data; S.S. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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