Peer Review Report

Review Report on Tween 80™-Induced Changes in Fatty Acid Profile of Selected Mesophilic Lactobacilli

Original Research, Acta Biochim. Pol.

Reviewer: Margaret Lorraine Britz
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EVALUATION

Q 1 **Please summarize the main findings of the study.**

This manuscript reports the fatty acid (FA) profiles detected by gas chromatography/mass spectrometry (GC-MS) for six species (11 strains) of lactic acid bacteria (LAB) formerly in the genus Lactobacillus (and still called by the trivial name lactobacilli), investigating the impact on FAs detected following growth on solid media (MRS agar) either containing or lacking the non-ionic surfactant Tween 80. The overall conclusions were that the presence of Tween 80 alters the distribution of extracted FAs, as determined from evaluating the relative proportion (%) of each FA in replicated extracts and the ratio of saturated/unsaturated FAs plus the ratio of the two main cyclopropyl derivatives of oleic and vaccenic acids, viz. dihydrosterculic/cis-9,10-methyleneoctadecanoic acid and lactobacillic/cis-11,12-methyleneoctadecanoic acid, respectively. The authors note that the data reported here differs from prior published reports and hence is a novel contribution to understanding the impact of exogenous FAs on cellular lipids.

Q 2 **Please highlight the limitations and strengths.**

The value in this study is it:
• updates prior publications that date back almost 30 years (notably Johnsson et al. 1995, which reported FA profiles for several species of lactobacilli cultured with and without Tween 80 and drew the link between Tween 80 and formation of cyclic-FAs from oleic acid by differentiating between the isomeric form of the cyclic-FA detected, along the same lines as reported in the current submission);
• included 3 strains of L. plantarum, 3 of L. rhamnosus, and 3 'casei' group strains, broadening the number of strains that have been analysed previously in parallel;
• used GC-MS analysis which detected isomeric forms and minor FA components which may not have been noted previously, due to the analytical methods used;
• used statistical analysis to support claims of differences between the current work and prior publications, and to group data to demonstrate that all strains tested showed altered FA profiles in the presence of Tween 80 (novel);
• correctly criticizes specific pieces of earlier work which may not have differentiated between isomeric forms (particularly of cyclic-FAs) or incorrectly named FA peaks, and
• noted (in the Introduction) that several prior publications used different analytical methods (which may influence outcomes) and that environmental conditions (including stress) can alter FA profiles, particularly synthesis of cyclic-FAs and altered saturated/unsaturated ratios in response to stress.
The limitations include:
• editorial matters
• Scientific or conceptual matters, which fall into two broad area:
o Experimental design, methods used, and
o Underlying assumptions that are not fully addressed within the text.
These are addressed in the full review to the editor

Q 3 **Please comment on the methods, results and data interpretation. If there are any objective errors, or if the conclusions are not supported, you should detail your concerns.**

Covered in the review report.

An annotated pdf is provided with over 70 comments across the text, addressing matters relating to clarity of methods, presentation of results and data interpretation. The data would make a useful contribution to the literature but the text needs to be shortened and references updated with the broader literature on this subject, given that many of the references cited are dated.

Check List

Q 4 **Please provide your detailed review report to the editor and authors (including any comments on the Q4 Check List)**

Tween 80-Induced Changes in Fatty Acid Profile of Selected Mesophilic Lactobacilli

This manuscript reports the fatty acid (FA) profiles detected by gas chromatography/mass spectrometry (GC-MS) for six species (11 strains) of lactic acid bacteria (LAB) formerly in the genus Lactobacillus (and still called by the trivial name lactobacilli), investigating the impact on FAs detected following growth on solid media (MRS agar) either containing or lacking the non-ionic surfactant Tween 80. The overall conclusions were that the presence of Tween 80 alters the distribution of extracted FAs, as determined from evaluating the relative proportion (%) of each FA in replicated extracts and the ratio of saturated/unsaturated FAs plus the ratio of the two main cyclopropyl derivatives of oleic and vaccenic acids, viz. dihydrosterculic/cis-9,10-methyleneoctadecanoic acid and lactobacillic/cis-11,12-methyleneoctadecanoic acid, respectively. The authors note that the data reported here differs from prior published reports and hence is a novel contribution to understanding the impact of exogenous FAs on cellular lipids. The value in this study is it:
• updates prior publications that date back almost 30 years (notably Johnsson et al. 1995, which reported FA profiles for several species of lactobacilli cultured with and without Tween 80 and drew the link between Tween 80 and formation of cyclic-FAs from oleic acid by differentiating between the isomeric form of the cyclic-FA detected, along the same lines as reported in the current submission);
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• noted (in the Introduction) that several prior publications used different analytical methods (which may influence outcomes) and that environmental conditions (including stress) can alter FA profiles, particularly synthesis of cyclic-FAs and altered saturated/unsaturated ratios in response to stress.
The weaknesses include:
• editorial matters
o Tables 2-5 have the full FA data set for all strains/conditions. This is currently in a file labelled 'supplementary material' and not in the main text of the manuscript. What will the final format show?
o If the detailed data is in 'supplementary material', is there a better way to demonstrate the data in visual form within the manuscript? Prior publications have used bar diagrams to show the relative proportions of FAs and impact of environmental variables. This may be useful to show which FAs make up the major proportion of the total and how the profiles alter in response to growth +/- Tween 80, as well as differences between species.
o Table 2 has a footnote to define the abbreviations used for the FAs detected, whereas the text uses the common/trivial name for these, leading to constant referral to the footnote to see which FA species is being described in the text versus the Tables (despite great familiarity with the structures and names in the literature!). Please resolve this, and check whether some of the short-hand abbreviations in the table are the most appropriate (e.g. cycC19:0,cis-10,11 for lactobacillic/cis-11,12-methyleneoctadecanoic acid).

What is the difference between '18:1' and the other specified isomers? The footnote also lists '18:2, conjugated octadecadienoic acid' as well as several specific isomers. Presumable 18:2_CLA_1 to _4 are isomers of CLA but the structures were not determined? The footnote should define this.
o Tables 3-5 footnotes refer to Table 1 for abbreviations, should be Table 2.
o Abbreviations are sometimes not defined when first used in the text (e.g. MALDI-TOF).
o Repetition of information plus phrasing within and across sections: careful editing and shortening of the text is required to minimise phrases repeated across sections, being more summative is encouraged rather than verbosity.
Other minor editorial matters are noted on an annotated pdf file provided.
• Scientific or conceptual matters, which fall into two broad area:
o Experimental design, methods used, and
o Underlying assumptions that are not fully addressed within the text.
These matters are handled in detail below.
Experimental design, methods used
1. It has been documented in several publications (some of which are cited already in the text) that Tween 80 has a protective effect on lactobacilli (and other LAB species) exposed to stressors, such as exposure to bile salts, short- or long-term heat stress, acid shock, oxygen exposure etc, and this is due to incorporation of oleic acid and its derivates into lipid membranes so that the fluidity of the membrane is altered to support survival. There is also literature which shows that Tween 80 inhibits fatty acid synthesis so that oleic acid (and others?) is scavenged from external sources AND that there is a temporal element to this, so that cells exposed to stress change their FA profile across the growth cycle – the authors should be able to find the literature on this (Reitermayer et al 2018 has been cited in the text for L. plantarum, and is useful regarding transcriptomics showing Tween 80 impeding FA synthesis; other recent papers from 2018 to 2023 are available that confirm FA inhibition and temporal changes, as well as the older literature).
The biomass used for FA analysis came from growth on modified MRS agar plates either containing or lacking Tween 80, harvested from plates at a single time point. There is currently insufficient detail in the methods to understand how biomass was harvested (from single colonies, the entire plate?), and this may have a substantive impact on the results obtained. The biomass may have been at different growth phases or be summative of different growth phases across the plates, for example. Many prior studies used liquid cultures, some used solid media: the authors need to consider whether the noted differences detected in FA profiles were due to the growth conditions and harvesting at stationary/late stationary phase cells from plates. Would different results be seen if liquid media were used? Possibly, and this needs to be considered and discussed by the authors. Given that the data presented shows greater proportions of cyclic-FA than reported in other publications, one may conclude that the 24 h/37C biomass was under stress or in stationary phase, whereas other prior reports were based on quite different growth conditions.
The authors need to be very specific about how cultures were harvest from plates, for clarity.
2. Line 106 states 'anaerobic' conditions were used for growth. Lines 194-195 state that vaccenic acid is made under aerobic conditions (old literature) but the data shows vaccenic acid was detected in all strains. Please specify what 'anaerobic conditions'
3. GC-MS was performed for analysis but the details of how the various isomeric form were identified require further information. Currently, the standards used are cited but 'other' FA ester identification is based on 'literature data', which is not very informative (noting that the references here are rather old). Given the authors criticize others for poor methodology, it is important that their own methods are described fully, so there is high confidence that the names of FAs detected are correct.
4. The authors need to specify the grade and source of Tween 80 used, given that the label 'Tween 80' can only be used when 'Oleic acid, ≥58.0% (balance primarily linoleic, palmitic, and stearic acids)' (cited from Sigma to show that high purity Tween 80 meets the international standard) and there is batch-to-batch variation/supplier differences. Given that some of the FA profiles that show differences in palmitic and other FAs (notably CLAs) that may be found in Tween 80, this is important for interpreting what changes are attributable to bacterial physiology and what may be due to scavenging the other components in Tween 80.
Underlying assumptions that are not fully addressed within the text
1. Table 1 provides a list of strains used and where they were obtained. It is presumed that the names of these strains are accurate and that they have not be reclassified into different species more recently. This is important for the 3 'casei' strains (Table 3), where phylogenetic analysis has shown that many strains called 'casei' in the past literature should be reclassified as 'paracasei' – see Ghosh et al. Microorganisms 2019, 7(11), 487; https://doi.org/10.3390/microorganisms7110487, although even recent publications continue to

use the original nomenclature and some genomic databases have not been updated. Again, this is relevant as the authors later argue that differences in FA profile for the 3 strains tested could be used to differentiate between the two species. Data in Figure 1 shows that the 3 strains do not cluster, unlike the L. rhamnosus and L. plantarum examples, which may flag concerns about the original naming of the strains. A quick analysis of the phylogenetic relationship between the 'casei' group strains used would be helpful, although I could not find genome sequences for C-431. This may strengthen, or refute, any claim that FA profiles can differentiate between 'casei' strains, despite only 3 being tested here (not enough to draw inference?).
2. The authors mention synthesis of oleic acid (line 174) and infer that oleic acid is converted into vaccenic acid (lines 442-443). The literature support, with evidence, that oleic acid is not synthesized by the strains under investigation to any large extent and, indeed, the data presented shows that in the absence of Tween 80 the proportion of oleic acid is very low, indicating little 'synthesis' of this FA. There is also no evidence in the literature, or their data, that oleic acid is converted into vaccenic acid: very early publications claimed this but were refuted later. The presence of Tween 80 inhibits production of the many enzymes involved in fatty acid synthesis (the FAS system, which is common in bacteria), based on transcriptomic and proteomic analyses in several lactobacilli (and other) species including the ones under study here, which implies that oleic acid is scavenged into cells and vaccenic acid is synthesized de novo later in the growth cycle when the FAS system is then expressed.

The authors need to consider their analysis, and inference from this, in context of the above, and in context of the actual composition of Tween 80 as a mixture dominated by oleic acid attached to sorbitan core (which also has isomers present) (>58%) but can contain free oleic acid and other FAs attached to the core. For example, the observation that more CLAs were detected in Tween 80-cultured cells may simply come from scavenging the CLAs from the supplied Tween 80.
3. The authors refer to 'branching' or 'branched' FAs lines 178-188. This is a little confusing, as branched FAs normally refers to ones synthesized from a propyl-CoA initial template and not acetyl-CoA, through the FAS system – and presumably from amino acid breakdown to provide the propyl-CoA. Please review this phrasing, ad 'branched FA' normally refers to amino-acid derived FAS template FAs. Some publications refer to the cyclo-FAs as 'kinked' and some synonyms for Tween 80 may imply branching but current chemical nomenclature favours the description of these as noted in the footnote of Table 2.
4. Lines 192-196: state that vaccenic acid is made under aerobic conditions. There are two main pathways for making vaccenic acid and in the lactobacilli this is by the anaerobic pathway. Indeed, the growth conditions in the methods section state 'anaerobic'. Please look up some recent reviews on how unsaturated FAs are made and the different routes used (particularly in firmicutes). There also seems to some confusion in the argument that growth condition/oxygenation and detection of unsaturated FAs has been reported in the literature. Clarify please – also in lines 233-237.
5. Lines 261-262: the authors note that the sum of oleic/dihydrosterculic and vaccenic/lactobacillic acids gives similar ratios as reported earlier for casei-group species by other authors. The key difference between other reports and the current study is that the cyclic-FAs are a much larger proportion of the total AND that this therefore alters the ratio of unsaturated to saturated as the cyclic-FAs are included in the latter. What the current data may be showing is an artifact of how cultures were grown and stage of growth of harvest, as cyclic-FAs are markers of stress in LAB. The authors are advised to consider this and revise the discussion points/conclusions in light of this consideration.
6. Lines 275-279: there are reports in the literature for FA profiles of the 'casei-group'.
7. Lines 423-475 have many repetitious statements that attempt to draw conclusions about the observations reported in the manuscript. Some of the statement imply that oleic and vaccenic acids compete in some way for incorporation into cell lipids, and this is also mentioned in the Abstract. There is no biochemical evidence provided in this manuscript to support this contention/hypothesis and the data is based on a single time point of cell harvest. Data from other studies how that Tween 80 suppresses synthesis of FAs during the early stages of growth and that vaccenic acid is made later in the growth cycle, presumably when oleic acid scavenging has depleted the supply of this FA. This means that as cells grow, the composition of glycerol-lipids changes, due to vaccenic acid being available and the modification of the acyl units by methylation occurs in situ within lipids. The authors need to consider the dynamics of how and when these FAs are made and transformed, and this may alter their perspective on putting an argument regarding competition (which implies that vaccenic acid is made simultaneously with oleic acid scavenging – this could be the case but evidence from the literature is needed).
Overall

The submission is largely confirming observations in the literature. The key differences are in the relative proportions of the cyclic-FAs detected. This may be due to several reasons including how cells were cultured and when they were harvested. The authors are encouraged to shorten the manuscript so it is more summative, give consideration to the dynamics of FA synthesis and the underlying biochemistry of how and when the observed FAs occur in cellular lipids.

Q 5 **Is the English language of sufficient quality?**

Yes.

Q 6 **Is the quality of the figures and tables satisfactory?**

Yes.

Q 7 **Does the reference list cover the relevant literature adequately and in an unbiased manner?**

No.

Q 8 **Are the statistical methods valid and correctly applied? (e.g. sample size, choice of test)**

Yes.

Q 9 **Are the methods sufficiently documented to allow replication studies?**

No.

Q 10 **Are the data underlying the study available in either the article, supplement, or deposited in a repository? (Sequence/expression data, protein/molecule characterizations, annotations, and taxonomy data are required to be deposited in public repositories prior to publication)**

Yes.

Q 11 **Does the study adhere to ethical standards including ethics committee approval and consent procedure?**

Not Applicable.

Q 12 **Have standard biosecurity and institutional safety procedures been adhered to?**

Not Applicable.

QUALITY ASSESSMENT

Q 13	Originality	
Q 14	Rigor	
Q 15	Significance to the field	
Q 16	Interest to general audience	
Q 17	Quality of the writing	
Q 18	Overall quality of the study