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TITLE:

Isolation of Lipoprotein Particles from Chicken Egg Yolk for the Study of Bacterial Pathogen Fatty Acid Incorporation into Membrane Phospholipids

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SUMMARY:

This method provides a framework for studying incorporation of exogenous fatty acids from complex host sources into bacterial membranes, particularly *Staphylococcus aureus*. To achieve this, protocols for the enrichment of lipoprotein particles from chicken egg yolk and subsequent fatty acid profiling of bacterial phospholipids utilizing mass spectrometry are described.

ABSTRACT:

Staphylococcus aureus and other Gram-positive pathogens incorporate fatty acids from the environment into membrane phospholipids. During infection, the majority of exogenous fatty acids are present within host lipoprotein particles. Uncertainty remains as to the reservoirs of host fatty acids and the mechanisms by which bacteria extract fatty acids from the lipoprotein particles. In this work, we describe protocols for enrichment of low-density lipoprotein (LDL) particles from chicken egg yolk and determining whether LDLs serve as fatty acid reservoirs for *S. aureus*. This method exploits unbiased lipidomic analysis and chicken LDLs, an effective and economical model for the exploration of interactions between LDLs and bacteria. The analysis of *S. aureus* integration of exogenous fatty acids from LDLs is performed using high-resolution/accurate mass spectrometry and tandem mass spectrometry, enabling the characterization of the fatty acid composition of the bacterial membrane and unbiased identification of novel combinations of fatty acids that arise in bacterial membrane lipids upon exposure to LDLs. These advanced mass spectrometry techniques offer an unparalleled perspective of fatty acid incorporation by revealing the specific exogenous fatty acids incorporated into the phospholipids. The methods outlined here are adaptable to the study of other bacterial pathogens and alternative sources of complex fatty acids.

INTRODUCTION:

Methicillin-resistant *S. aureus* (MRSA) is the leading cause of healthcare-associated infection and the associated antibiotic resistance is a considerable clinical challenge[1-3]. Therefore, the development of novel therapeutic strategies is a high priority. A promising treatment strategy for Gram-positive pathogens is inhibiting fatty acid synthesis, a requirement for membrane phospholipid production that, in *S. aureus*, includes phosphatidylglycerol (PG), lysyl-PG, and cardiolipin[4]. In bacteria, fatty acid production occurs via the fatty acid synthesis II pathway (FASII)[5], which is considerably different from the eukaryotic counterpart, making FASII an attractive target for antibiotic development[5,6]. FASII inhibitors primarily target FabI, an enzyme required for fatty acid carbon chain elongation[7]. The FabI inhibitor triclosan is broadly used in consumer and medical goods[8,9]. Additional FabI inhibitors are being developed by several pharmaceutical companies for the treatment of *S. aureus* infection[10-26]. However, many Gram-positive pathogens, including *S. aureus*, are capable of scavenging exogenous fatty acids for phospholipid synthesis, bypassing FASII inhibition[27-29]. Thus, the clinical potential of FASII inhibitors is debated due to considerable gaps in our knowledge of the sources of host fatty acids and the mechanisms by which pathogens extract fatty acids from the host[27,28]. To address these gaps, we developed an unbiased lipidomic analysis method to monitor incorporation of exogenous fatty acid from lipoprotein particles into membrane phospholipids of *S. aureus*.

During sepsis, host lipoprotein particles represent a potential source of host-derived fatty acids within the vasculature, as a majority of host fatty acids are associated with the particles[30]. Lipoproteins consist of a hydrophilic shell composed of phospholipids and proteins that enclose a hydrophobic core of triglycerides and cholesterol esters[31]. Four major classes of lipoproteins--chylomicron, very low-density lipoprotein, high-density lipoprotein, and low-density lipoprotein (LDL)—are produced by the host and function as lipid transport vehicles, delivering fatty acids and cholesterol to and from host cells via the vasculature. LDLs are abundant in esterified fatty acid including triglycerides and cholesterol esters[31]. We have previously demonstrated that highly purified human LDLs are a viable source of exogenous fatty acids for PG synthesis, thus providing a mechanism for FASII inhibitor bypass[32]. Purifying human LDLs can be technically challenging and time consuming while commercial sources of purified human LDLs are prohibitively expensive to use on a routine basis or to perform large-scale bacterial screens. To address these limitations, we have modified a procedure for the enrichment of LDLs from chicken egg yolk, a rich source of lipoprotein particles[33]. We have successfully used untargeted, high-resolution/accurate mass spectrometry and tandem mass spectrometry to monitor incorporation of human LDL-derived fatty acids into the membrane of *S. aureus*[32]. Unlike previously reported methods, this approach can quantify individual fatty acid isomers for each of the three major staphylococcal phospholipid types. Oleic acid (18:1) is an unsaturated fatty acid present within all host lipoprotein particles that is readily incorporated into *S. aureus* phospholipids[29,30,32]. *S. aureus* is not capable of oleic acid synthesis[29]; therefore, the quantity of phospholipid-incorporated oleic acid establishes the presence of host lipoprotein-derived fatty acids within the staphylococcal membrane[29]. These phospholipid species can be identified by the state-of-the-art mass spectrometry method described here, offering unprecedented resolution of the membrane composition of *S. aureus* cultured in the presence of a fatty acid source it likely encounters during infection.

PROTOCOL:

NOTE: The following protocol for enrichment of LDL particles from chicken egg yolk is derived from Moussa et al. 2002[33].

1. Preparation of chicken egg yolk for enrichment of LDL particles

1.1. Sanitize two large chicken eggs by washing the shells with 70% ethanol solution and allow to air dry.

1.2. Sanitize the egg separator using 70% ethanol solution and allow to air dry. Attach the egg separator onto the lip of a medium sized beaker.

1.3. Crack each egg individually into the egg separator and allow the albumen to flow into the beaker. The intact egg yolk will be retained by the separator.

1.4. Wash the egg yolk twice with 30 mL of sterile phosphate-buffered saline (PBS) to remove residual albumen.

1.5. Gently place the egg yolk onto filter paper.

1.6. Puncture the vitelline membrane with a sterile pipette tip and drain the contents of the membrane into a sterile 50 mL conical centrifuge tube. Discard the membrane and filter paper.

2. Fractionation of LDL-containing plasma from chicken egg yolk

2.1. Add approximately two volumes of 0.17 M NaCl at pH 7.0 to the egg yolk and mix vigorously. Then mix this solution at 4 °C for 60 min.

2.2. Centrifuge the egg yolk dilution at 10 °C at 10,000 x *g* for 45 min. Remove the plasma fraction (supernatant) from the granular fraction (pellet) into a sterile 50 mL conical tube.

2.3. Repeat step 2.2.

3. Isolation of LDL particles from plasma

3.1. Mix the plasma fraction with 40% ammonium sulfate (w/v) at 4 °C for 60 min.

3.2. Adjust the pH of the plasma fraction with a 420 mM NaOH solution to 8.7.

3.3. Centrifuge the egg yolk dilution at 4 °C at 10,000 x *g* for a duration of 45 min. Remove the upper semisolid yellow fraction into 7 kDa pore size dialysis tubing. Provide room in the tubing to allow for it to swell.

3.4. Dialyze overnight at 4 °C in 3 L of ultrapure water to remove the ammonium sulfate. Gently agitate the water using a stir bar.

3.5. Transfer dialyzed solution into a sterile 50 mL conical centrifuge tube.

3.6. Centrifuge the solution at 4 °C at 10,000 x *g* for a duration of 45 min. Carefully remove the upper semisolid yellow fraction to a sterile tube and store at 4 °C.

4. **Assessment of chicken LDLs as a source of fatty acids**

4.1. Subculture *S. aureus* cells into 5 mL of fatty acid-free 1% tryptone broth and incubate overnight at 37 °C with shaking (225 rpm). For fatty acid auxotrophs, supplement cultures with a source of fatty acids.

4.2. Dilute overnight cultures to an optical density (OD) at 600 nm (OD_{600}) of 0.1 in 1% tryptone broth. Pipette 50 µL of the cell suspension into each well of a round-bottom 96-well plate.

NOTE: When working with fatty acid auxotrophs, wash the overnight cultures in two volumes of tryptone broth and resuspend in 5 mL of tryptone broth to limit carryover of fatty acids before determining the OD of the culture.

4.3. For the wells containing untreated controls, add 50 µL of 1% tryptone broth per well.

4.4. To the wells containing the experimental cell suspensions, add 50 µL of 1% tryptone broth supplemented with 10% egg yolk-derived LDL, 2 µM triclosan, or a mixture of 10% egg yolk-derived LDL and 2 µM triclosan.

NOTE: At this point, each well will contain 100 µL and the final concentration of egg yolk-derived LDL and triclosan per well will be 5% and 1 µM, respectively.

4.5. Measure OD_{600} over time, using a microplate reader set at 37 °C with continuous, linear shaking to monitor growth.

5. Incubation of *S. aureus* with LDLs for membrane lipid analysis.

5.1. Culture an isolated colony into 5 mL of fatty acid-free 1% tryptone broth and incubate overnight at 37 °C with shaking (225 rpm).

5.2. Dilute overnight cultures 1:100 into a sterile 250 mL baffled flask containing 50mL of 1% tryptone broth. Incubate to mid-log phase (approximately 4 h) at 37 °C with shaking.

5.3. Transfer 25 mL of culture to a sterile 50 mL centrifuge tube and pellet the cells. Remove the supernatant and resuspend the cell pellet in 750 µL of 1% tryptone broth.

5.4. Combine the resuspended cells and aliquot 300 µL of the cell suspension into a sterile 1.5 mL centrifuge tube.

5.5. Add LDLs to the desired final concentration and incubate at 37 °C with shaking (225 rpm) for 4 h.

5.6. Centrifuge the cultures at 4 °C at 16,000 x *g* for a duration of 2 min and wash the cell pellets in two volumes of sterile PBS then repeat.

5.7. Record the weight of each wet cell pellet. Snap-freeze the cell pellets on dry ice or in liquid nitrogen and store at -80 °C or proceed directly to section 6.

6. **Extraction of *S. aureus* membrane lipids**

6.1. Place frozen *S. aureus* cell pellets on dry ice. Add 0.5 mm zirconium oxide beads on top of each cell pellet, using a volume of beads approximately equal to the volume of the cell pellet.

NOTE: As an alternative to this method of lipid extraction, researchers can use the well-established Bligh and Dyer or Folch methods for exacting lipids from bacterial cells[34].

6.2. Add 740 µL of 75% methanol (HPLC grade) chilled to -80 °C directly to the cell pellets.

6.3. Add 2 µL of 50 µM dimyristoyl phosphatidylcholine (prepared in methanol) per 1 mg of cells as an internal standard. Close the test tube and place the 1.5 mL centrifuge tubes containing each sample into an available port in a Bullet Blender tissue homogenizer. Homogenize the samples on low speed, setting 2-3, for 3 min.

6.4. Visually inspect the samples for homogeneity. If clumps of cells are visible, continue homogenization in the Bullet Blender in 2 min increments.

6.5. Remove the samples from Bullet Blender and transfer to a chemical fume hood.

6.6. Add 270 µL of chloroform to each sample tube. Vortex the samples vigorously for 30 min.

CAUTION: Chloroform is a possible carcinogen.

6.7. Centrifuge the samples in a benchtop centrifuge for up to 30 min at a minimum 2000 x *g*. Faster speeds may be used with compatible centrifuge tubes and the duration of centrifugation may be shortened to 10 min.

6.8. In a chemical fume hood, collect the monophasic supernatant and transfer to a new test tube, while carefully avoiding the protein pellet at the bottom of the extraction tube.

6.9. Add 740 µL of 75% methanol (HPLC grade) and 270 µL of chloroform to the protein pellet,

and re-extract each sample as described in steps 6.6-6.8 above. Combine the supernatant from the second extraction with the previously collected supernatant for each sample.

6.10. Evaporate the extraction solvents under a stream of inert gas such as nitrogen or argon, or under vacuum using a centrifuge concentrator (**Table of Materials**).

6.11. Wash dried lipid extracts three times with 1.0 mL of aqueous 10 mM ammonium bicarbonate solution and re-dry the samples as in step 6.10.

6.12. Resuspend the dried lipid extracts in a suitable nonpolar solvent such as isopropanol. Resuspend samples using 20 µL per 1 mg of fresh cell weight determined in step 5.7 Alternatively, if the weight of the cell pellets is unknown, resuspend the samples in 200 µL of isopropanol and proceed to Section 7.

7. Analysis of *S. aureus* lipid profiles using high resolution/accurate mass spectrometry

7.1. Prior to conducting a full lipid analysis, select representative test samples from the experimental group(s) and analyze them over a range of sample dilution factors to determine sample dilution ranges in which the total lipid concentrations fall within the linear range of detector response for the mass spectrometer, as previously described[35].

7.2. Evaporate aliquots of each sample lipid extract to be subjected to lipid analysis, by drying the aliquots under inert gas or under vacuum in a centrifuge concentrator (**Table of Materials**).

7.3. Resuspend each dried lipid extract in liquid chromatography–mass spectrometry (LC-MS) grade isopropanol:methanol (2:1, v:v) containing 20 mM ammonium formate, using volumes equivalent to an optimal sample dilution factor as determined in step 7.1.

7.4. For an untargeted lipid analysis, samples may be introduced directly to the high resolution/accurate mass spectrometry platform without the use of chromatography by flow injection or direct infusion of extracts[35,36]. Transfer the diluted lipid extracts prepared in step 7.3 to an appropriate autosampler vial or 96-well plate.

7.5. For flow injection-based analysis, place the autosampler vials into a temperature-controlled (15 °C) autosampler of an HPLC system capable of capillary/low flow applications, such as a HPLC (**Table of Materials**) equipped with an electronic flow proportioning and flow monitoring system.

7.6. Fill the HPLC solvent reservoirs with LC-MS grade isopropanol:methanol (2:1, v:v) containing 20 mM ammonium formate.

7.7. Using Agilent Chemstation software, program the HPLC autosampler to perform 5 µL sample injections. From the **Instrument** menu, select **Set Up Injector**, and type **5.0** in the Injection Volume field. The units are given as microliters. Ensure that the HPLC is set to isocratic

flow at 1 µL per min of 2:1 (v:v) isopropanol:methanol containing 20 mM ammonium formate.

7.8. From the Chemstation **Instrument** menu, select **Set Up Pump…** and then select the **Micro Flow** mode toggle switch.

7.9. In the **Timetable** fields, enter: **Time 0.00, 100% B, Flow 1.0**. Hit **Enter** and create a second row in the **Timetable** by entering **Time 10.0, 100% B, Flow 1.0**. Select the **OK** button at the bottom of the **Set Up Pump** menu. These settings will enable 10 analytical runs at a flow rate of 1.0 µL per minute.

7.10. Introduce eluate from the HPLC transfer line to the mass spectrometer using an electrospray ionization source fitted with a low-flow (34 G) metal needle.

7.11. Using Thermo Tune Plus instrument control software, select the **Setup** menu and select **Heated ESI Source**. Set the ionization voltage to 4000 V and sheath gas to 5 (arbitrary units) by typing these values into the corresponding fields of the dialogue box. Similarly, set the Capillary Temp to 150 °C and the S-lens to 50%.

NOTE: These values need to be optimized for each mass spectrometry platform.

7.12. For untargeted lipid analysis, use a high resolution/accurate mass MS platform (**Table of Materials**) as the detector.

7.13. Using Thermo Tune Plus software, click the **Define Scan** button, and in the **Analyzer** menu select **FTMS**. Set the **Mass Range** field to **Normal** and in the **Resolution** field select **100,000**. Ensure that **Scan Type** is set to **Full**. Under the **Scan Ranges** menu, enter **200** in the **First Mass (m/z)** field, and enter **2000** in the **Last Mass (m/z) field**.

7.14. Ensure that negative polarity is utilized to detect the most abundant *S. aureus* lipids.

7.15. Repeat the sample analysis using ion mapping tandem mass spectrometry (MS/MS) fragmentation of all lipid ions within a spectral region of interest in order to confirm lipid structures and fatty acid constituents. Alternatively, selected lipid ions of interest may be subjected to MS/MS analysis after initial lipid identifications have been assigned in Section 8.

8. Database searching to identify endogenous *S. aureus* and exogenous LDL-derived lipids

8.1. Use Thermo Xcalibur software to further refine observed mass accuracy. In Xcalibur, under the **Tools** menu, select the **Recalibrate Offline**. After the **Recalibrate Offline** window opens, load the mass spectrum file to be recalibrated by selecting the **File** menu and selecting the **Open** option.

8.2. Open the file of interest, toggle the **Insert Row** button at the top of the view window, to view the total ion chromatogram for the MS run. Average the acquired MS signals by left-clicking

the computer mouse on one edge of the observed signal peak in the total ion chromatogram and dragging the mouse across the broadest part of the peak.

8.3. Under the **Scan Filter** menu, select the filter corresponding to full scan MS data. Load a reference file containing the theoretical monoisotopic masses of at least three known *S. aureus* endogenous lipids by selecting the **Load Ref…** button and selecting the reference file. Check the **Use** box next to each lipid monoisotopic mass.

8.4. Click the **Search** button at the bottom of the viewing window. Re-average the MS signal across the signal peak in the total ion chromatogram as done previously.

8.5. Click the **Convert** button near the bottom of the viewing window. When the **Convert** dialogue box opens, click **OK**. Omit this step if data was collected on mass spectrometry platforms from vendors other than Thermo Scientific.

8.6. Using Xcalibur software, export the recalibrated accurate mass peak lists for each untreated or LDL-treated sample to separate worksheets of an Excel file. Select the **Qual Browser** icon. Open the recalibrated file of interest by selecting the **File** menu and selecting the **Open…** option.

8.7. Average the signal across the broad peak in the total ion chromatogram as described in step 8.2.

8.8. Right click the thumbtack icon in the mass spectrum viewing window and select **View** | **Spectrum List**. From the same menu, select **Display Options** and then **All Peaks** toggle box in the **Display** menu. Click the **OK** button to close the viewing window.

8.9. Right click the thumbtack icon in the mass spectrum viewing window again and select the **Export** | **Clipboard (Exact Mass)**. Paste the exported data cell A1 of the first worksheet into a new Excel spreadsheet.

8.10. Delete the first 8 rows of text in the exported data file, such that cell A1 of the Excel spreadsheet contains the first mass data point from the mass spectrum. Repeat the exporting of each recalibrated MS file, using a new worksheet in the Excel file for each exported peak list.

8.11. Using the Lipid Mass Spectral Analysis (LIMSA) software[37] Add-In for Excel, construct a database containing molecular formulas of known *S. aureus* lipid species as described by Hewelt-Belka et al. 2014[38], as well as formulas representing lipid species that could hypothetically be present in LDL.

NOTE: Additionally, care should be taken to include potential molecular formulas in the database for hypothetical bacterial lipids that have incorporated major LDL fatty acids, such as oleic (18:1) and linoleic (18:2) fatty acids[32].

8.12. To construct the database, open a blank Excel spreadsheet. In cell A1 of the first worksheet, type the theoretical/computed monoisotopic mass of the lipid species to be added to the database, corresponding to the mass of the lipid species in the ionic state observed in the mass spectrometer. In cell B1, enter a name for the lipid species, such as PG(34:0).

8.13. In cell C1, enter the molecular formula for the lipid species, corresponding to the ionic state of the lipid observed in the mass spectrometer. In cell D1, enter the charge of the lipid species as observed in the mass spectrometer. Move to cell A2 to begin a new entry for the next lipid species to be entered in the searchable database.

8.14. Repeat the steps 8.12 and 8.13 until all desired lipid species have been entered into the database. Save the database file and leave it open in Excel.

8.15. In Excel, select the **Add-Ins** menu in. Select **LIMSA** to start the LIMSA software. From the main menu, click the **Compound Library…** button. In the new window that appears, click **Import Compounds**. This will upload the compound database for use by the LIMSA software.

8.15. Perform accurate-mass based lipid identifications on all MS spectra using the LIMSA software Add-In for Excel according to the vendor's instructions[35]. From the LIMSA main menu, select **Peak List** under the **Spectrum type** menu. Select **Positive mode** or **Negative mode** to correspond with the polarity in which the MS data was acquired.

8.16. In the **Peak fwhm (m/z)** window, enter the desired mass search window for peak finding. A mass tolerance search window of 0.003-0.005 m/z is recommended for high resolution/accurate mass MS data.

8.17. In the **Sensitivity** window, enter the desired baseline cutoff (for example, 0.01% relative abundance). In the **Isotope correction** menu, select **Linear fit** or **Subtract** algorithms, either of which may be used with peak list data.

8.18. Highlight lipid compounds to include in the database search by clicking on desired lipid species within the **Available Compounds** window. Click the **Add** button to add highlighted lipid species to the search group.

8.19. Define internal standards by clicking on added compounds, then changing the **Concentration** window to the corresponding concentration of the selected internal standard.

8.20. Ensure that the internal standard and selected lipid species to be quantitated belong to the same class name by selecting each added lipid species and internal standard and typing a class name (such as PG or Lipid) in the Class field.

8.21. To save the searchable group of lipid compounds for future use, click the **Save** button next to the **Groups** menu.

8.22. Ensure that the Excel file containing all exported MS peak lists is open to the sheet corresponding to the first *S. aureus* MS run, and then click the **Search** button from the LIMSA main menu.

NOTE: The output of the LIMSA database search will include a list of mass spectral features matched to lipids present in the database constructed in steps 8.12-8.13, as well as concentrations for each matched feature following normalization to one or more selected internal standards.

8.23. Use Xcalibur software to examine accurate mass MS/MS spectra for m/z corresponding to lipid ions of interest in order to confirm fatty acid constituents present in each identified lipid molecular species. Select the **Qual Browser** icon. Open the MS/MS file of interest by selecting the **File** drop-down menu and selecting the **Open…** option.

8.24. Select the scan filter corresponding to MS/MS analysis of a lipid m/z of interest by right mouse-clicking on the thumbtack icon in the mass spectrum viewing window and select **Ranges** from the menu. In the new window that appears, select the **Filter** menu to select the scan.

8.25. Average the signal across the total ion chromatogram as described in step 8.2. Use MetaboAnalyst (www.metaboanalyst.ca) software to perform appropriate statistical tests. Evaluate statistically significant difference in *S. aureus* lipid composition by comparing normalized lipid abundances across untreated and LDL-treated conditions.

REPRESENTATIVE RESULTS:

The protocol for the enrichment of LDL from chicken egg yolk is illustrated in **Figure 1**. This process begins by diluting whole egg yolk with saline and separating the egg yolk solids referred to as granules from the soluble or plasma fraction containing the LDLs (**Figure 1**)[33]. The LDL content of the plasma fraction is further enriched by precipitation of the ~ 30-40 kDa β-livetins (**Figure 2**)[33]. The presence of protein bands at 140, 80, 65, 60 and 15 kDa correlate with the apoproteins of LDLs (**Figure 2**)[33,39]. Treatment with triclosan inhibits growth of *S. aureus* in fatty acid-free media[32]. We have previously demonstrated that supplementing cultures with egg yolk plasma or purified human LDLs as exogenous fatty acid sources overcomes triclosan-induced growth inhibition (**Figure 3**)[32]. Similarly, supplementation of triclosan-treated cultures with enriched egg yolk LDL restores growth (**Figure 3**). Further, addition of egg yolk LDLs support the growth of a previously characterized *S. aureus* fatty acid auxotroph (**Figure 4**)[32]. For the most accurate mass spectrometry-based profiling of *S. aureus* incorporation of exogenous fatty acids, it is important to limit the presence of free fatty acids in the growth medium. The free fatty acid composition of 1% tryptone broth and chicken egg yolk LDLs diluted in tryptone broth was determined by employing flow injection high-resolution/accurate mass spectrometry and found minimal quantities of free fatty acid (**Figure 5**). The same untargeted mass spectrometry analysis was performed to determine the fatty acid composition of *S. aureus* phospholipids after exposure to chicken egg yolk LDLs. Orthogonal partial least-squares discriminant analysis (OPLS-DA)[40] of abundant *S. aureus* membrane phospholipids demonstrated clear class separation of untreated and chicken egg yolk LDL-treated conditions, as shown in the OPLS-DA scores plot (**Figure 6A**).

The OPLS-DA loadings plot indicated numerous phosphatidylglycerol species as important variables in the PLS-DA model. Notably, phospholipids containing unsaturated fatty acids, a molecular marker of exogenous fatty acid incorporation, are enriched in the LDL supplemented cultures compared to cells incubated in the absence of LDLs (**Figure 6B**). Previous studies have found that chicken egg yolks are a rich source of unsaturated fatty acids with oleic acid (18:1) being the most abundant[41,42]. In agreement with these observations, we found oleic acid to be the most common unsaturated fatty acid utilized for phospholipid synthesis when *S. aureus* cultures were supplemented with chicken egg yolk LDLs (**Figure 6C**). **Table 1** further illustrates that the fatty acid profiles of membrane phospholipids are altered when *S. aureus* is grown in the presence of egg yolk LDL.

FIGURE AND TABLE LEGENDS:

Figure 1: An illustration of LDL enrichment from chicken egg yolk utilizing centrifugation and ammonia sulfate precipitation. (**A**) The reagents necessary for the enrichment of LDL from chicken egg yolk. (**B**) The flow chart depicts the significant steps of the LDL enrichment process.

Figure 2: Protein profile of chicken egg yolk prior to and after enrichment for LDL. Protein lysates were prepared using RIPA buffer. Protein lysate (15 µg) was loaded into an 8% acrylamide SDS-PAGE gel. Gels were stained overnight with Bio Rad Protein reagent. The molecular weights in kDa of LDL associated proteins are denoted along the right side of the image. M: protein marker, Y: chicken egg yolk, and LDL: chicken egg yolk LDL enrichment

Figure 3: Egg yolk-derived LDLs protect *S. aureus* from triclosan-induced FASII inhibition. The growth of *S. aureus* was monitored over time via measurement of OD_{600} in 1% tryptone broth in the following conditions: 1% tryptone broth (TB), 1 µM triclosan (TCS), 1 µM triclosan with 1% egg yolk plasma (TCS + EYP), 5% egg yolk LDL (LDL), or 1 µM triclosan with 5% egg yolk LDL (TCS + LDL). The mean from three independent experiments is shown. Error bars represent the standard deviation of the mean.

Figure 4: Growth of a *S. aureus* fatty acid auxotroph is supported by egg yolk-derived LDL. The growth of a fatty acid auxotroph in 1% tryptone broth (TB) with or without 5% egg yolk LDL (LDL) supplementation was monitored over time via measurement of OD_{600}. The mean from three independent experiments is shown. Error bars represent the standard deviation of the mean.

Figure 5: Free fatty acid content measured in 1% tryptone broth or chicken egg yolk LDL. Free fatty acids were detected by flow injection high-resolution/accurate mass spectrometry and tandem mass spectrometry. Normalized numbers of ions per mg of protein was determined for 1% tryptone broth and 1% tryptone broth supplemented with 5% chicken egg yolk LDLs.

Figure 6: Chicken egg yolk low-density lipoproteins are a reservoir of exogenous fatty acids for synthesis of *S. aureus* phosphatidylglycerol. (**A**) Scores plot of orthogonal partial least-squares discriminant analysis of chicken egg yolk LDL-treated and untreated *S. aureus* membrane phospholipids identified using high resolution/accurate mass spectrometry. (**B**) Percentage of unsaturated phosphatidylglycerol (UPG) compared to total membrane PG of *S. aureus* grown in

the absence (WT) or presence (WT + LDL) of chicken egg yolk LDLs. (**C**) Unsaturated fatty acid (UFA) profile of membrane PG of *S. aureus* grown without (WT) or with (WT + LDL) chicken egg yolk LDLs graphed as a percentage of the amount of total PG fatty acids.

Table 1: Fatty acid profile of *S. aureus* cultured in the presence of chicken egg yolk LDLs. We used an unbiased lipidomic analysis utilizing high-resolution/accurate MS and MS/MS to determine the fatty acid profile of *S. aureus* PG. *S. aureus* was incubated in the presence or absence of chicken egg yolk LDLs, and the PG profile of these cells was compared to that of cells cultured in 1% tryptone broth.

DISCUSSION:

S. aureus incorporates exogenous fatty acids into its membrane phospholipids[27,32,43]. Phospholipid synthesis using exogenous fatty acids bypasses FASII inhibition but also alters the biophysical properties of the membrane[27,32,44]. While incorporation of exogenous fatty acids into phospholipids of Gram-positive pathogens is well documented, gaps remain in the identity of host fatty acid reservoirs and the structural alterations to each of the three major staphylococcal phospholipid types that result from exogenous fatty acid incorporation. Here, we describe protocols which can be employed to: i) enrich LDL particles from chicken egg yolk, a source of fatty acids, ii) determine the effects of exogenous fatty acids on the growth of *S. aureus*, and iii) utilize an unbiased lipidomic analysis for monitoring exogenous fatty acid incorporation into membrane phospholipids of *S. aureus*. The advanced mass spectrometry method provided in this study offers an extraordinary perspective of the membrane composition of *S. aureus* grown in the presence of exogenous fatty acids.

Several Gram-positive pathogens utilize exogenous fatty acids for membrane synthesis and, as with *S. aureus,* the possible sources of exogenous fatty acids during infection are poorly understood[27,43]. The growth analysis described here can be modified to evaluate the proliferation of other Gram-positive pathogens in the presence of lipoproteins if the growth kinetics of each pathogen are considered. Additionally, other complex host sources of exogenous fatty acids could be tested using this protocol if the potential effects of the fatty acid source on background optical density are controlled. Moreover, the described mass spectrometry method for analysis of bacterial lipids is sufficiently flexible to enable lipidome evaluation from virtually any bacterial species. As lipid accurate mass data is collected in 'full scan' MS mode, little *a priori* knowledge of the lipid content of the bacterial species of interest is required, unlike targeted analytical methods based on known fragmentation patterns of specific lipids[32,45,46]. In the 'untargeted' analytical workflow we describe, the downstream data analysis, and particularly the searching of accurate mass peak lists against a lipid database, are key steps that are highly adaptable and may support a broad range of bacterial species and experimental treatments. When constructing or choosing a searchable database to enable identification of lipid species, researchers must consider a wide range of hypothetical endogenous lipid species, while also allowing for detection of novel or unforeseen exogenous lipids derived from the experimental treatment.

In the present study, a high resolution/accurate mass spectrometer (**Table of materials**) was employed due to its ultra-high resolution/accurate mass capabilities. Alternatively, numerous

other high resolution/accurate mass spectrometry platforms could be successfully implemented to perform untargeted lipid analysis. Similarly, a wide range of sample introduction methods including direct infusion, desorption electrospray ionization, or matrix-assisted laser desorption ionization, that enable direct analysis of lipid extracts, could be utilized to rapidly collect untargeted lipidomic data. The inclusion of liquid chromatography prior to sample introduction, when used in combination with high resolution/accurate mass spectrometry, may permit the resolution of some isobaric lipid species during full-scan MS data collection. However, the inclusion of chromatography necessitates ensuring that the chromatographic method of choice is versatile enough to enable separation and detection of unanticipated or novel lipid species that may be present following experimental treatments. Database searching to identify lipids present in the dataset may be performed using any publicly available searchable database. While LIMSA software enables facile development of user-defined databases of tens of thousands of hypothetical lipid species, numerous other options exist for identifying lipids from high resolution/accurate mass MS peak lists. The LIPID MAPS consortium (www.lipidmaps.org) provides tools for searching computational and experimental databases of hypothetical lipids using high resolution/accurate MS-generated peak lists within a user-defined mass tolerance, and many software vendors offer their own solutions for analyzing lipidomics data.

Successful growth curve and exogenous fatty acid analysis is dependent on several factors including LDL purity and limiting background fatty acid levels. Proper identification of the LDL-containing fraction is crucial. The above protocol and **Figure 1** illustrate the correct fraction to retain for each step of the enrichment process. We have had success with the use of 40% ammonium sulfate (purity ≥99.5%) for the precipitation and subsequent removal of β-livetins. However, others have reported that the purity and concentration of ammonia sulfate added to the egg yolk plasma can significantly impact this step[47]. Limiting ammonium sulfate contamination in the LDL enrichment is important for the LDL preparation to be used in bacterial assays, as high concentrations of ammonium sulfate can restrict growth[48]. During dialysis, ample free space in the dialysis tubing must be provided to allow for the diffusion and removal of the ammonium sulfate. Further optimization of the dialysis process may include additional water changes during the overnight incubation. We have found overnight dialysis to provide the best results, although Moussa et al. report dialysis of 6 h is sufficient. It is critical that the starting concentration of cells in the growth curves are kept consistent between trials. For *S. aureus,* diluting cells to an initial OD_{600} of 0.05 has provided the most consistent results. Additionally, high concentrations of FASII inhibitors can result in non-specific effects on bacterial cells. For example, triclosan concentrations above 7 µM induce cytoplasmic membrane damage in *S. aureus,* therefore it is necessary that the concentration of this compound remain below this level[49]. We have found a final triclosan concentration of 1 µM results in reproducible growth assays. When evaluating a potential source of exogenous fatty acids for bacterial phospholipid synthesis, it is important to minimize the fatty acid contribution of the culture medium. In the above assays, the culture medium of 1% tryptone supports adequate growth of *S. aureus* and has minimal fatty acid contamination[29,32].

Limiting background fatty acid levels is particularly important for downstream mass spectrometry-based fatty acid profiling. Others have reported the quantity of free fatty acids in

chicken egg yolk is naturally low[41] and our analysis supports this conclusion (**Figure 5**). Using tryptone broth and thoroughly washing cells with PBS after incubation are essential. Additionally, it is important to consider the growth phase of the cells. We chose mid-log phase cells to ensure ample bacterial phospholipid synthesis. Other potential contaminating sources of exogenous fatty acids may be introduced after bacterial growth, such as during the lipid extraction steps or subsequent sample preparation prior to mass spectrometry analysis[50]. Exogenous fatty acids introduced at any step of sample preparation could be detected as free fatty acids during mass spectrometry analysis. Baking laboratory glassware in a high temperature oven (at least 180 °C) overnight can remove exogenous fatty acids from test tubes used for lipid extraction and lipid storage. Additionally, laboratory supplies including plastics may be rinsed with methanol to reduce fatty acid background[50]. Residual fatty acids from previous analyses may also contaminate internal surfaces of the mass spectrometer itself. Inclusion of analytical blanks during mass spectrometry analysis for determination of background levels of free fatty acids is therefore strongly advised.

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DISCLOSURES:

The authors have no disclosures.

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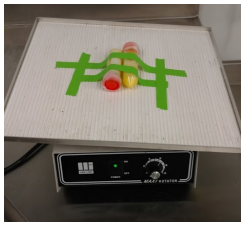
Figure 1

A

B

1. Prepare egg yolk and saline suspension.



2. Mix egg yolk suspension for 1 h at 4 °C.

3. Centrifugate and remove the plasma fraction.

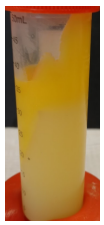
4. Add 40% ammonium sulfate (w/v) to plasma and mix for 1 h at 4 °C.

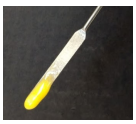
5. Adjust pH of mixture to ~8.7 with NaOH and centrifugate.

6. Remove the upper semisolid layer and transfer to dialysis tubing.

7. Dialyze in water overnight at 4 °C.

8. Transfer dialyzed solution to a centrifuge tube.





Semisolid layer

9. Centrifugate and transfer the upper yellow semisolid layer to a centrifuge tube.

10. Dilute in sterile PBS and store at 4 °C.

Figure 2

Click here to access/download;Figure;Figure 2.ai

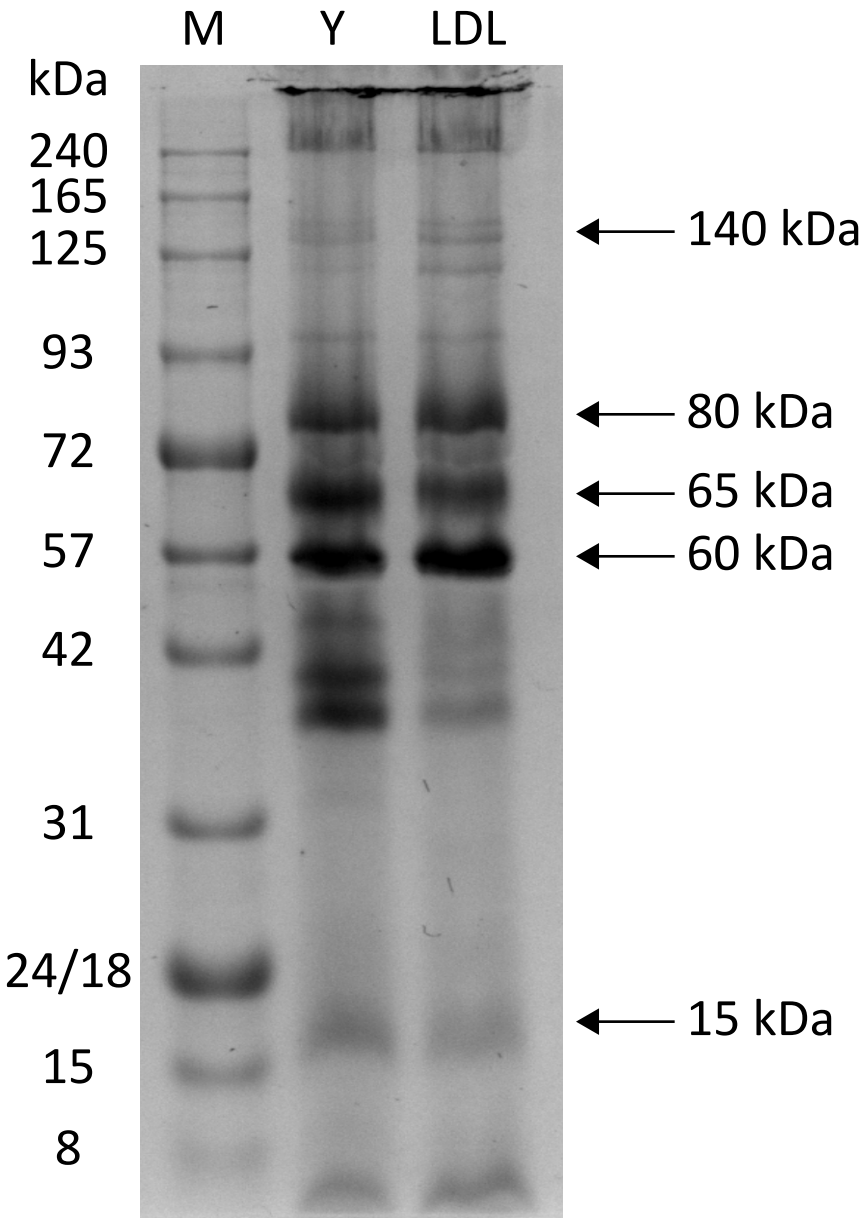


Figure 3

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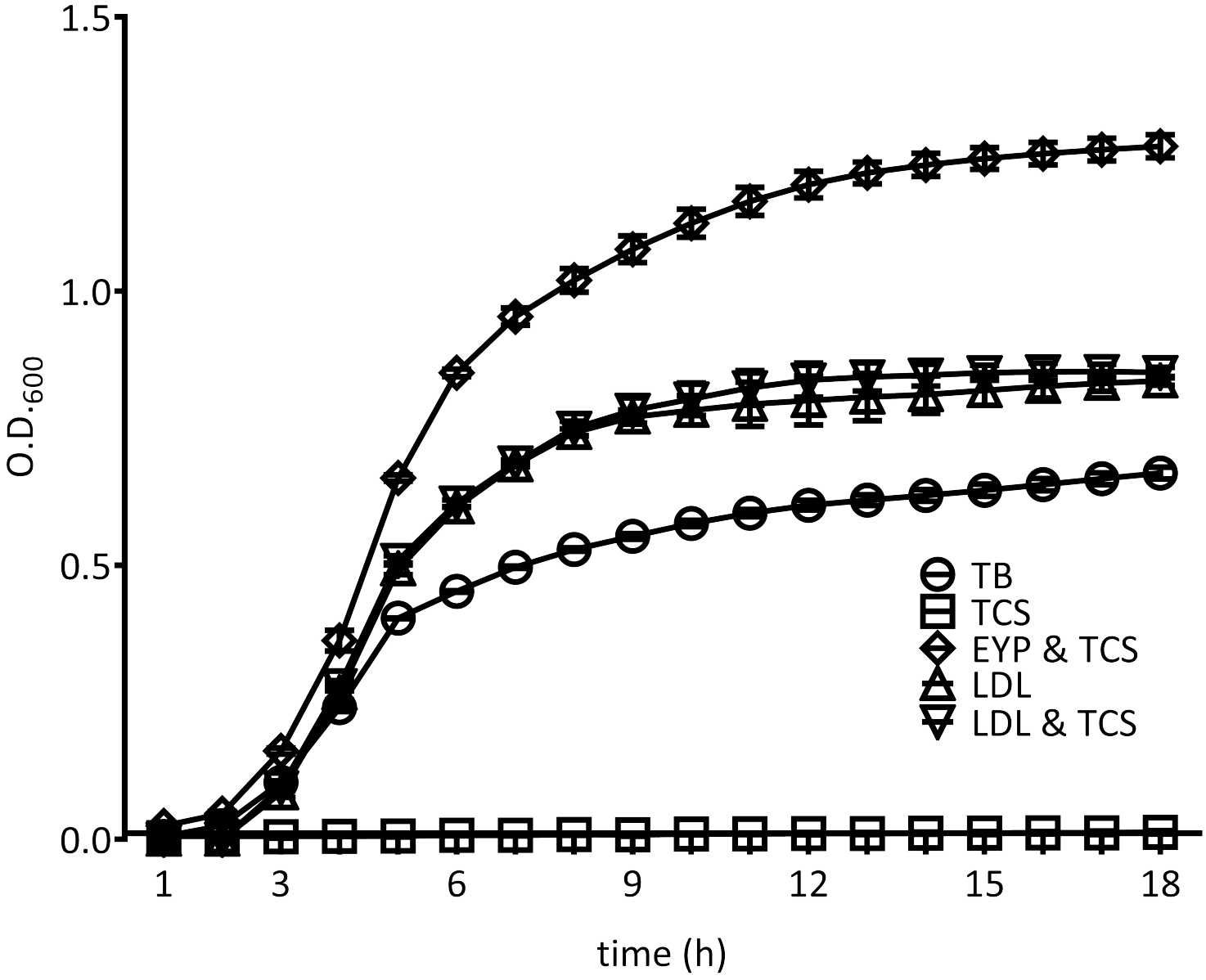

Figure 4

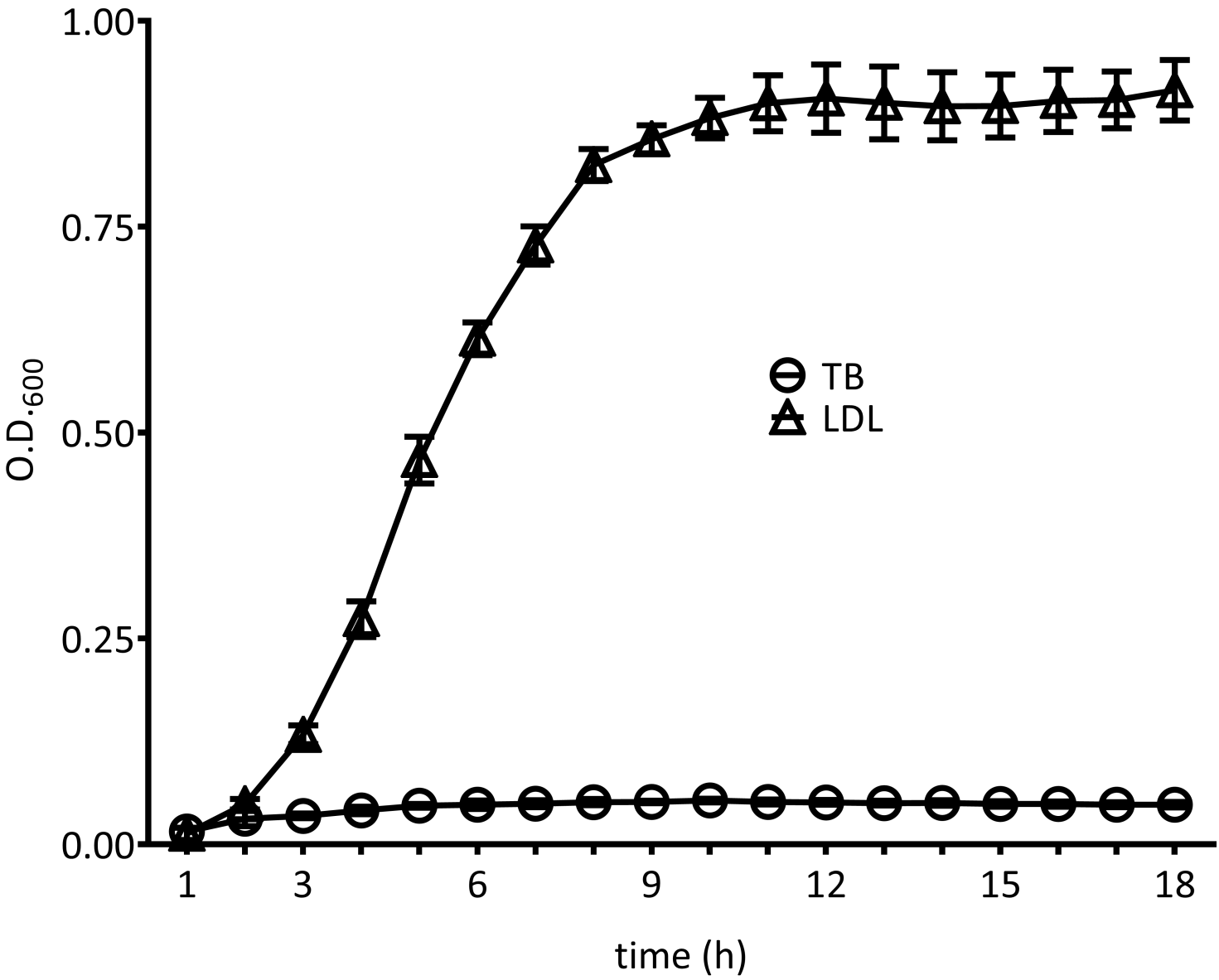

Figure 5

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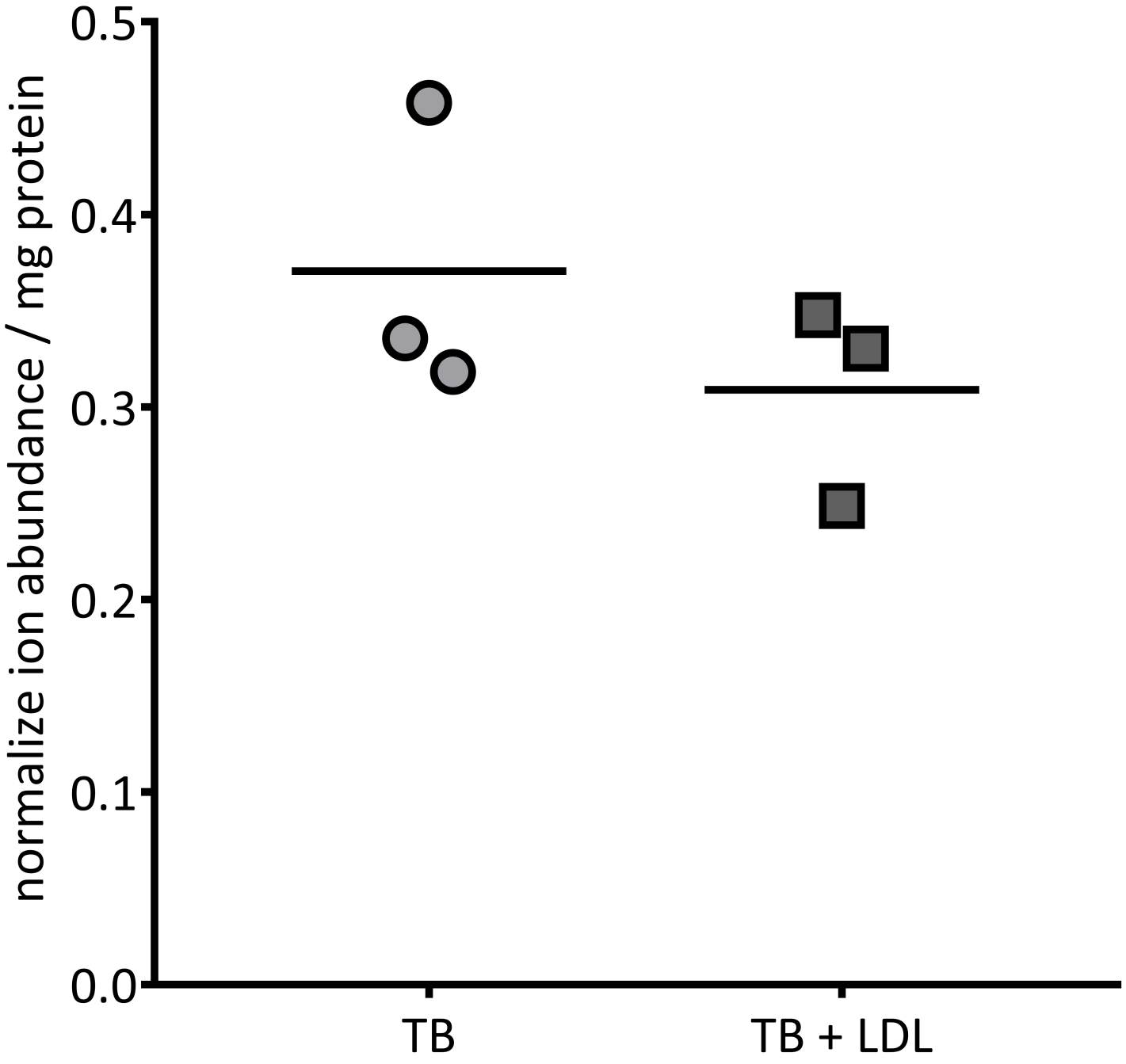

Figure 6

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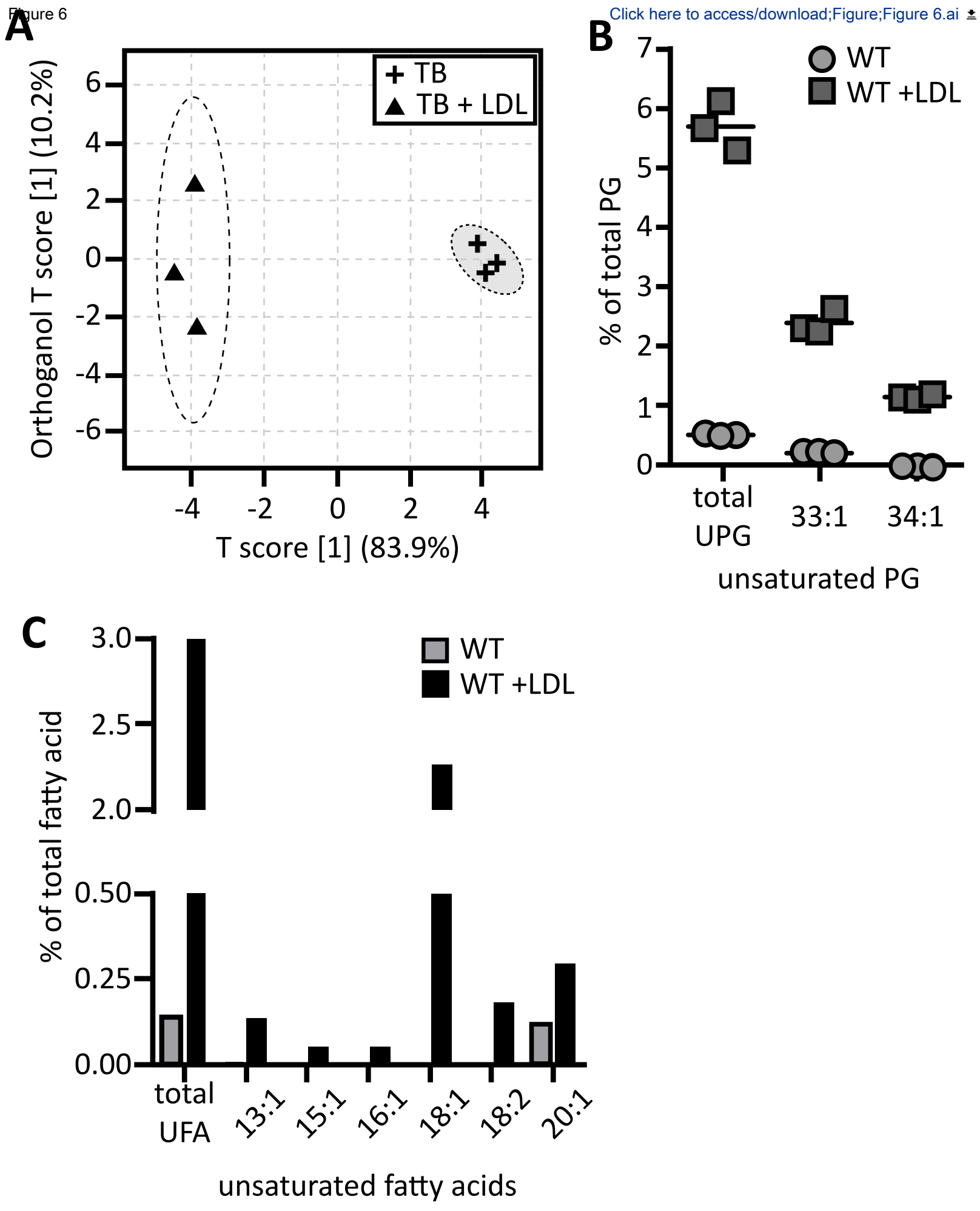

Table 1: Fatty acid profile of *S. aureus* cultured in the presence of chicken egg yolk LDLs

	WT cultured in tryptone broth			
Phosphatidyl glycerol (TC:TDB)[a]	Normalized ion abundance/mg of protein	SD	Fatty acids[c]	Normalized ion abundance/mg of protein
24:0	0	0	ND[b]	0.052031116
26:0	0	0	ND	0.009539117
28:0	0.127937113	0.04528	15:0_13:0	0.167643281
28:1	0.006765427	0.00157	ND	0.002776821
30:0	8.680180809	2.68375	15:0_15:0	14.04873592
30:1	0	0	ND	0.010152161
31:0	4.150511117	1.31658	16:0_15:0, 14:0_17:0	10.17590926
31:1	0.016156004	0.01216	13:1_15:0, 12:1_19:0	0.473478683
32:0	29.29259262	8.82993	15:0_17:0	48.24342037
32:1	0.02074815	0.00941	ND	0.307044942
33:0	9.000460122	2.78194	18:0_15:0, 16:0_17:0	15.4531776
33:1	0.162934812	0.04796	ND	2.921832928
33:2	0	0	ND	0.167492702
34:0	12.3064043	3.70242	19:0_15:0, 17:0_17:0	18.40129157
34:1	0	0	ND	1.423605186
34:2	0.000470922	0.00082	ND	0.156133734
35:0	5.727462455	1.74583	20:0_15:0, 18:0_17:0	7.771538992
35:1	0.17337586	0.05727	20:1_15:0	0.772202525
35:2	0	0	ND	0.038758757
36:0	0.671004303	0.2116	21:0_15:0, 19:0_17:0	0.967295024
36:2	0	0	ND	0.495485065
36:3	0	0	ND	0.059268233
37:0	0.060466411	0.01961	22:0_15:0, 20:0_17:0	0.114526894
38:2	0	0	ND	0.079469521

[a]Detected as $[M-H]^-$ ions. TC, total chain length; TDB, total number of double bonds.

[b]ND, not determined

[c]Fatty acids are listed in order of isomer abundance. An underscore between fatty acid designations as tandem mass spectrometry alone cannot rule out the possibility that lipid species exist as a mixtur

WT cultured in tryptone broth supplemented with LDLs

SD	Fatty Acids[c]
0.02677	ND
0.00362	ND
0.02392	15:0_13:0
0.00372	15:0_13:1
2.4531	15:0_15:0
0.00449	15:1_15:0, 13:1_17:0
1.88431	16:0_15:0, 14:0_17:0, 18:0_13:0
0.09063	13:1_15:0, 18:1_13:0, 12:1_19:0
8.95664	15:0_17:0, 16:0_16:0
0.07305	18:1_14:0, 16:1_16:0
2.98171	18:0_15:0, 16:0_17:0
0.30851	18:1_15:0
0.03211	18:1_15:1, 18:2_15:0
3.21385	19:0_15:0, 17:0_17:0
0.20066	18:1_16:0
0.03929	18:2_16:0
1.28515	20:0_15:0, 16:0_19:0, 18:0_17:0
0.08526	20:1_15:0, 18:1_17:0
0.01481	18:2_17:0, 18:1_17:1
0.2572	21:0_15:0, 20:0_16:0, 19:0_17:0, 22:0_14:0
0.04473	18:1_18:1, 18:2_18:0
0.02291	18:2_18:1, 20:3_16:0, 20:2_16:1
0.01852	22:0_15:0, 20:0_17:0, 18:0_19:0
0.02872	18:2_20:0, 16:1_20:1

indicates that each fatty acid may be present in either the SN1 or SN2 position,
e of positional isomers.

Name of Material/ Equipment	Company	Catalog Number
Ammonium sulfate	Fisher	BP212R-1
Cell culture incubator	Thermo	MaxQ 6000
Centrafuge	Thermo	75-217-420
Costar assay plate	Corning	3788
Filter paper	Schleicher & Schuell	597
Large chicken egg	N/A	N/A
Microplate spectrophotometer	BioTek	Epoch 2
NaCl	Sigma	S9625
S. aureus strain AH1263	N/A	N/A
Dialysis tubing	Pierce	68700
Tryptone	Becton, Dickison and Company	211705
0.5 mm zirconium oxide beads	Next Advance	ZROB05
Bullet Blender	Next Advance	BBX24B
Methanol (LC-MS grade)	Fisher	A4561
Chloroform (reagent grade)	Fisher	MCX10559
Isopropanol (LC-MS grade)	Fisher	A4611
Dimyristoyl phosphatidylcholine	Avanti Polar Lipids	850345C-25mg
Ammonium bicarbonate	Sigma	9830
Ammonium formate	Sigma	70221-25G-F
Xcalibur software	Thermo Scientific	OPTON-30801
LTQ-Orbitrap Velos mass spectrometer	Thermo Scientific	
Agilent 1260 capillary HPLC	Agilent	
SpeedVac Vacuum Concentrators	Thermo Scientific	

Comments/Description
≥99.5% pure
Sorvall Legen XTR, rotor F14-6x250 LE
96 well
Common store bought egg
Provided by Alex Horswill of the University of Colorado
7,000 MWCO
≥99.5% pure
high resolution/accurate mass MS

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Dear Dr. Wu,

Thank you for providing Editorial Comments, we have addressed all 18 comments in the attached revised manuscript. Additionally, the reviewers made excellent suggestions that we also addressed. Below is our point-by-point response to each comment. We thank the reviewers for taking the time to assess our manuscript.

Reviewer #1 Concerns:

No major concerns noted.

Minor Concerns:

1. It is likely that for many users, the mass spectrometry analyses would be conducted by a core facility. Therefore, while the methodological description may still be a valuable resource, the description of lipoprotein extraction from egg yolk will likely be more useful to JoVE readers.

We agree with Reviewer #1's assessment that enrichment of LDL from chicken egg yolk is the more pertinent aspect of our manuscript. However, the advantage of describing the mass spectrometry is that it provides a resource for researchers to obtain a deeper understanding of the technique beyond submission to a core facility, if they so choose.

2. Section 4, "Bacterial growth assessment of LDL preparation" does not include any specific instructions related to growth with exogenous lipoprotein supplement. For example, instruction line 4.2 simply states that overnight cultures should be diluted to an OD of 0.05, but does not specify that the cells should be diluted into 1% Tryptone broth, or Tryptone broth containing 5% egg yolk lipoprotein.

We added this information to Step 4.2 (lines 154-158) and thank the reviewer for noting this omission.

3. Section 6; "Extraction of S. aureus membrane lipids" line 6.1 could specify the mass of beads to be added. This section might also mention that although use of beads is your preferred method, that this may not be strictly necessary since a number of studies continue to cite the classic method of Bligh and Dyer. Alternately, explain why you believe that your protocol is superior.

We comment that the Bligh and Dyer method is an alternative to our lipid extraction protocol (Step 6.1, lines 195-198), giving readers access to another acceptable approach for extracting lipids.

Reviewer #2 Concerns:

No major concerns noted.

Minor Concerns:

1. In the Abstract it is more correct to say fatty acids incorporated into the phospholipids rather than complexed within the phospholipids - this is what the mass spec shows after all.

We have made the correction (line 42) and thank the reviewer for noting this improvement.

2. I don't know whether it is necessary to mention this but egg yolk agar is used to demonstrate lipase activity in Clostridium species. It's interesting, just saying.

This is an excellent point and agree that the use of chicken egg yolk as a diagnostic for bacterial pathogen lipase activity is fascinating. However, this property of chicken egg yolk is beyond the scope of this manuscript. It would also be interesting to know if Clostridium species incorporate chicken egg yolk-derived fatty acids into their membrane lipids. We mention that our protocol can be used to monitor fatty acid incorporation in other bacterial species in the discussion.

In total, we appreciate the reviewers' excellent comments and feel that they have contributed to much improved manuscript. Please contact me if there is any additional information you require.

Sincerely,

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