The Effect of Dietary Synbiotic on Bone Mass and Mechanical Strength in Rats during Simulated Weightlessness

William M. Berry, Jonathan H. Berry, Yudhisthir Paudel

Colorado State University-Pueblo, Pueblo, CO 81001, USA
Advisers: Dr. Annette Gabaldon and Dr. Jude Depalma
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Abstract
Long term exposure to a weightless or low gravity environment leads to a rapid decrease in bone mineral density known as spaceflight osteopenia. This is due to the absence of gravitational loading on the skeletal structure. This study examines the effectiveness of dietary supplementation with probiotics to counteract the lack of gravity and thus the disruption of normal intestinal microbiota that is a hypothesized contributor to bone density loss. Forty adult male rats were studied under four different conditions in a 2 x 2 factorial design with main effects of diet (control and added probiotics/prebiotics) and two weight (simulated weightlessness and control). Hindlimb unloading was performed at all times for 14 days followed by 14 days of recovery (reambulation). The synbiotic diet consisted of Lactobacillus acidophilus and Lactococcus lactis in a powdered prebiotic frutoooligosaccharide vehicle. The hindlimb bones (femur, tibia) harvested from rats kept at Idaho State University. The following measurements are made by graduate students at CSU-Pueblo: 1) mechanical strength properties using a 3-point bending tester made by students on campus for this purpose; 2) bone volume and density determination; 3) bone composition and mineral density; and 4) bone specific mineral profiles using ICP mass spectrophotometry.

1. Introduction
One of the most important characteristics to consider on spaceflight is zero-gravity. Prolonged exposure to low-gravity environments has the tendency to increase spaceflight osteopenia on otherwise fit astronauts. In order to combat these symptoms, different diets supplemented with prebiotic/probiotic elements are to be tested to examine whether bone structure and integrity can be promoted or supported when gravitational loading is not present. This purpose of this study is to determine if a synbiotic diet can be an effective preventative measure or treatment to prevent loss of bone mineral density and to prevent increased bone fragility that can both occur under low-gravity conditions. Low bone mineral density applies, not only to astronauts, but to humans dealing with bone loss and osteoporosis, particularly late in age. In this study, the methodology and outcomes of executing three-point bending test of the rat bone femurs is examined and analyzed. By percentage the two composing elements of bone are predominately collagen type I and hydroxyapatite crystals. Research done by J.D. Curry states that, “The organic matrix, predominately type I collagen, provides strength and flexibility to bone and also determines its structural organization. The mechanical properties of bone are dependent upon the properties of these constituents (Curry 137)”. Curry’s position assumes that the
organic matrix of the bone, specifically collagen type I, is crucial for the tensile strength of the bone.

II. Materials and Methods

a. Biological

40 male Spague-Dawley adult rats were kept at Idaho State University, which is where the hindlimb bones (femur and tibia) were collected. They were kept frozen at -70°C; the bones were wrapped in gauze-soaked saline to keep them hydrated during storage both at Idaho State University and Colorado State University in Pueblo. The bones were studied under four different conditions in a 2 x 2 factorial design with main effects of diet: control and added probiotics/prebiotics, and weight: control and simulated weightlessness. These were classified as loaded control (LC), unloaded control (ULC), loaded synbiotic (LS), and unloaded synbiotic (ULS). The synbiotic diet consisted of Lactobacillus acidophilus and Lactococcus lactis in a powdered prebiotic fructooligosaccharide vehicle.

b. Engineering

Due to budgetary constraints, it was decided that the three point testing unit was to be manufactured on-site at CSU-Pueblo. The testing device was built to specifications as defined by a specific set of guidelines governing all such experiments, ASAE Standard: ASAE S459, which sets the standards for the procedures which must be followed if an experiment is to be considered valid. Per these standards, the three point bend test must be performed using equipment that is reproducible to +/- 1%.

The ASAE specifications for the test equipment stated that the ends of the supports and the breaking tip had to be rounded, with a radius of 4 mm. This step required the use of CSU-Pueblo’s CNC vertical milling machine. These components were successfully created and properly integrated with the device.

The device constructed is designed to be load-applying with deflection control, utilizing an electronic load cell and a displacement indicator. The measurement of the displacement was to be accomplished with a digital surface and depth gauge with a total spindle travel of just over an inch, ample travel distance for measuring the displacement of something as small as a rat's femur. The final design called for the meter to be mounted above the ram so that it could measure the total distance traveled between the initial contact of the breaking tip with the bone and the actual breaking of the bone. A
load cell is mounted in-line with the ram to measure force components (in Newtons) of the test.

After the machining was completed, the equipment was assembled and tested. A variable power supply was used to power the motor, in order to fine-tune the desired deflection speed. Test materials (small pieces of wood) were originally placed in the equipment and broken to ensure that the motor was capable of supplying sufficient torque to meet the requirements and the load cell was capable of reliably reading the amount of force required. Different voltage settings were tested and the feed rate was determined by using the surface gauge to determine the distance moved by the ram and a clock to measure the time. It was found that the desired rate of feed could be achieved at a setting of just over seven volts. With the equipment tested and functionality verified, it was turned over to the individuals that were going to perform the experiment.

c. Integration and Interfacing

In order to properly record and analyze data obtained from the testing unit, the deflection indicator and the load cell were interfaced using Matlab and Excel to establish device connections and log data (See Appendix (c)). Upon initialization of the project, new sensors needed to be purchased, since older devices used were deemed inadequate to properly and effectively interface with any kind of electronic data logging. Upon receiving the new device, new programming code also had to be written to accommodate it. Using available code and with the help of instructors at CSU-Pueblo, an interface was successfully used to record deflection and load cell data. With the added interface, the testing unit is capable of running multiple tests with very good repeatability.

III. Testing

The test sample or femur was slowly thawed at room temperature and was kept wrapped in the saline-soaked gauzes up until testing. The three point testing machine, as described earlier, was set at a span of 20 millimeters. A 1.0 Newton preload was applied to the test sample to take the slack out of the machine. The three-point test machine was run at constant rate of 0.2 millimeters per second until critical failure of the femur. After each test the data was compiled in an Excel file for final analysis. The equation to calculate inertia ($I$), the elastic (Young’s) modulus ($E$) and fracture strain ($\sigma$) is:

$$I = \frac{\pi (BH^3 - bh^3)}{64}$$

where $B$ and $H$ are the maximum and the minimum external diameter, respectively, and $b$ and $h$ are the maximum and minimum internal diameter, respectively.
\[ E = \frac{FL^3}{48If} \]  

(2)

\[ \text{Slope} = \frac{F}{f} \]  

(3)

\[ \sigma = \frac{FLC}{4l} \]  

(4)

where \( F \) is force, \( L \) is the span, \( C \) is the distance from the cross-sectional center of mass, and \( f \) is the deflection.

During the experimental data analysis of elastic (Young’s) modulus of the 40 femurs, there were several graph types encountered. As seen below there were 3 common graph types and the range in which the slope was taken (See Appendix (a) 1a). From these areas, the above equations were used to calculate Young’s modulus.

IV. Results

After performing multiple tests, it appears that the average elastic modulus differs statistically from one set to the next. Upon testing, some values were encountered when testing the loaded control bones where the elastic modulus appeared to be much higher than the other tested values (See Appendix (b) 2a-2b). In order to account for these anomalies, two separate comparisons are done, one including the data points and the other without. However, the loaded control bones are expected to naturally exhibit much greater elastic modulus and fracture strain values, and has no effect on the comparison between the unloaded control and unloaded synbiotic specimens.

V. Conclusion

The unloaded synbiotic specimens had a tendency to exhibit increased elastic modulus and fracture strain values when compared to the unloaded control specimens. According to the recorded data and calculations, the unloaded synbiotic diets were able to raise these values by approximately thirteen percent (see Appendix (a) 1b-1c). Higher values for elastic modulus and fracture strain may be indicative that the effects of spaceflight osteopenia may be reduced by the introduction of the modified prebiotic/probiotic diets.

VI. References


VII. Appendices

Appendix (a)
Specimen Readouts and Values

Fig. 1a. Fracture types encountered during testing
Fig. 1b. Average elastic modulus values (outliers included)

Average Elastic Modulus

Fig. 1c. Average elastic modulus values (outliers excluded)

Average Elastic Modulus
Fig. 1d. Fracture strain values (outliers excluded)

Fig. 1e. Fracture strain values (outliers included)
Appendix (b)
Data History

Fig. 2a. Numerically sorted test data (outliers excluded)

Fig. 2b. Numerically sorted test data (outliers included)
Appendix (c)
Matlab Programming Interface Code

function varargout = attempt1(varargin)
% ATTEMPT1 M-file for attempt1.fig
% ATTEMPT1, by itself, creates a new ATTEMPT1 or raises the existing
% singleton*.
% H = ATTEMPT1 returns the handle to a new ATTEMPT1 or the handle to
% the existing singleton*.
% ATTEMPT1('CALLBACK',hObject,eventData,handles,...) calls the local
% function named CALLBACK in ATTEMPT1.M with the given input
% arguments.
% ATTEMPT1('Property','Value',...) creates a new ATTEMPT1 or raises
% the existing singleton*. Starting from the left, property value pairs
% are applied to the GUI before attempt1_OpeningFcn gets called. An
% unrecognized property name or invalid value makes property
% application stop. All inputs are passed to attempt1_OpeningFcn via varargin.
% *See GUI Options on GUIDE's Tools menu. Choose "GUI allows only
% one instance to run (singleton)".
% See also: GUIDE, GUIDATA, GUIHANDLES
% Edit the above text to modify the response to help attempt1
% Last Modified by GUIDE v2.5 05-Mar-2010 14:02:43
% Begin initialization code - DO NOT EDIT

gui_Singleton = 1;
if nargin && ischar(varargin{1})
    gui_State.ischar(varargin{1})
    gui_State.gui_Callback = str2func(varargin{1});
end

if nargin
    [varargout{1:nargout}] = gui_mainfcn(gui_State, varargin{:});
else
    gui_mainfcn(gui_State, varargin{:});
end
% End initialization code - DO NOT EDIT
% --- Executes just before attempt1 is made visible.
function attempt1_OpeningFcn(hObject, eventdata, handles, varargin)
% This function has no output args, see OutputFc
% hObject handle to figure
% eventdata reserved - to be defined in a future version of MATLAB
% handles structure with handles and user data (see GUIDATA)
% varargin command line arguments to attempt1 (see VARARGIN)
% Choose default command line output for attempt1
handles.output = hObject;
% Update handles structure
guidata(hObject, handles);
% UIWAIT makes attempt1 wait for user response (see UIRESUME)
uiwait(handles.figure1);
% --- Outputs from this function are returned to the command line.
function varargout = attempt1_OutputFcn(hObject, eventdata, handles)
% varargout cell array for returning output args (see VARARGOUT);
% hObject handle to figure
% eventdata reserved - to be defined in a future version of MATLAB
% handles structure with handles and user data (see GUIDATA)
% Get default command line output from handles structure
varargout{1} = handles.output;
% --- Executes on button press in begintest.
function begintest_Callback(hObject, eventdata, handles)
% hObject handle to begintest (see GCBO)
% eventdata reserved - to be defined in a future version of MATLAB
% handles structure with handles and user data (see GUIDATA)
% 3-point test machine code, using Mitutoyo indicator and
% Loadstar iLoad Mini load cell.
instrreset; % resets any open ports
loadcell = serial('COM3','Timeout',0.4);
indicator = serial('COM6','Timeout',0.4);
set(loadcell,'BaudRate',9600); %Make sure COM ports are set
set(indicator,'BaudRate',2400); % correctly with Hyperterminal
set(loadcell,'Parity','none'); % before using.
set(indicator,'Parity','none'); %Even though both devices are
set(loadcell,'DataBits',8); %operating at different bitrates,
set(indicator,'DataBits',8); %they are sampled by the program
set(loadcell,'StopBits',1); % at the same rate, so no
set(indicator,'StopBits',1); %number compensation is needed.
set(loadcell,'FlowControl','software');
set(indicator,'FlowControl','none');
set(loadcell,'Terminator','CR');
set(indicator,'Terminator','CR');
fopen(loadcell); %Opens USB pathway and accesses load cell
fopen(indicator); %Opens USB pathway and accesses linear indicator
% ********** data acquisition section **********
cmd = 'o0w1'; % "o0w1" asks load cell for 1 reading. "o0w0" asks for
% a continuous stream from the cell, until a carriage
% return is sent. "ct0" will zero the load cell.
cr = char(13);lf = char(10);crlf = strcat(cr,lf);
cmd = strcat(cmd,cr); % also works with only CR or only LF
handles.force = [];
% Establishes vector for load cell data
cmd = strcat(cmd,cr); % also works with only CR or only LF
hold on
for i = 1:400 % (i) is number of data points the program will collect.
default should be 400
    fprintf(loadcell, cmd);
a = fscanf(loadcell, '%d')
handles.force(i) = a; % placing load cell data into matrix (load)
fprintf(indicator, cmd);
a = fscanf(indicator, '%c', 3); % (3) is removing excess characters from
% indicator readout, to make plotting % easier.
a = str2num(fscanf(indicator, '%c'))
handles.distance(i) = a; % placing indicator data into matrix (distance)
plot(handles.distance, handles.force)
end
hold off
toc
fclose(loadcell); % closes connection to load cell
fclose(indicator); % closes connection to indicator
instrreset; % resets serial ports after use.
% Update handles structure
guidata(hObject, handles);

% --- Executes on button press in export.
function export_Callback(hObject, eventdata, handles)
% hObject    handle to export (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
c = [handles.distance', handles.force']
xlswrite(handles.filenamer, c);
% Update handles structure
guidata(hObject, handles);

% --- Executes on button press in preloadstream.
function preloadstream_Callback(hObject, eventdata, handles)
% hObject    handle to preloadstream (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
if handles.z == 1
    clc
    instrreset;
    % resets any open ports
    loadcell = serial('COM3', 'Timeout', 0.4);
    set(loadcell, 'BaudRate', 9600); %Make sure COM ports are set
    set(loadcell, 'Parity', 'none'); % correctly with Hyperterminal
    set(loadcell, 'Databits', 8); %Even though both devices are set
    set(loadcell, 'StopBits', 1); %operating at different %they are sampled by the program
    set(loadcell, 'FlowControl', 'software'); %at the same rate, so no
    %number compensation is needed.
    set(loadcell, 'Terminator', 'CR');
    fopen(loadcell); %Opens USB pathway and accesses load cell % **************************** data acquisition section ****************************
    cmd = 'o0w1'; % "o0w1" asks load cell for 1 reading. "00w0" asks for
    % a continuous stream from the cell, until a carriage
% return is sent. "ct0" will zero the load cell.
cr = char(13);lf = char(10);crlf = strcat(cr,lf);
cmd = strcat(cmd,cr);  % also works with only CR or only LF
% Establishes vector for load cell data acquisition  % Establishes
vector for indicator data acquisition

tic
while handles.z  % (i) is number of data points the program will
collect.
    fprintf(loadcell, cmd);
    a = fscanf(loadcell,'%d')
    % placing indicator data into matrix(distance)
end
toc
fclose(loadcell);  % closes connection to load cell
% closes connection to indicator
instrreset;  % resets serial ports after use.
end
% Hint: get(hObject,'Value') returns toggle state of preloadstream

% --- Executes on button press in preload_toggle.
function preload_toggle_Callback(hObject, eventdata, handles)
% hObject    handle to preload_toggle (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
% Hint: get(hObject,'Value') returns toggle state of preload_toggle
handles.z = get(hObject,'Value');
% Update handles structure
gdata(hObject, handles);
function load_data_Callback(hObject, eventdata, handles)
% hObject    handle to load_data (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
% Hints: get(hObject,'String') returns contents of load_data as text
%        str2double(get(hObject,'String')) returns contents of load_data
% as a double
% --- Executes during object creation, after setting all properties.
function load_data_CreateFcn(hObject, eventdata, handles)
% hObject    handle to load_data (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    empty - handles not created until after all CreateFcn
% called
% Hint: edit controls usually have a white background on Windows.
%       See ISPC and COMPUTER.
if ispc && isequal(get(hObject,'BackgroundColor'),
    get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end
function filenamer_Callback(hObject, eventdata, handles)
% hObject    handle to filenamer (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
handles.filenamer = get(hObject,'String');

% Update handles structure
guidata(hObject, handles);
% Hints: get(hObject,'String') returns contents of filenamer as text
% str2double(get(hObject,'String')) returns contents of filenamer as a double
% --- Executes during object creation, after setting all properties.
function filenamer_CreateFcn(hObject, eventdata, handles)
% hObject    handle to filenamer (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    empty - handles not created until after all CreateFcns called
% Hint: edit controls usually have a white background on Windows.
%       See ISPC and COMPUTER.
if ispc && isequal(get(hObject,'BackgroundColor'),
                get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end
# Appendix (d)

## Bone Measurements and Characteristics

### HLU Study #2: Group - LC "Loaded Control"; i.e. weight on all 4 limbs; regular rat chow diet (no synbiotics)

**Rat Femur Bone Midshaft Diameter Measurements (Right Leg)**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Wet weight (g)</th>
<th>B (M-L) O.D. mm</th>
<th>b (M-L) I.D. mm</th>
<th>D (A-P) O.D. mm</th>
<th>d (A-P) I.D. mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-31 femur</td>
<td>1.30</td>
<td>4.35</td>
<td>2.85</td>
<td>3.63</td>
<td>2.27</td>
</tr>
<tr>
<td>LC-32 femur</td>
<td>1.23</td>
<td>4.56</td>
<td>3.45</td>
<td>2.94</td>
<td>1.93</td>
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<tr>
<td>LC-33 femur</td>
<td>1.35</td>
<td>4.74</td>
<td>3.14</td>
<td>2.72</td>
<td>2.58</td>
</tr>
<tr>
<td>LC-34 femur</td>
<td>1.29</td>
<td>4.43</td>
<td>3.32</td>
<td>3.31</td>
<td>2.21</td>
</tr>
<tr>
<td>LC-35 femur</td>
<td>1.31</td>
<td>4.32</td>
<td>2.93</td>
<td>3.84</td>
<td>2.49</td>
</tr>
<tr>
<td>LC-36 femur</td>
<td>1.41</td>
<td>4.47</td>
<td>2.97</td>
<td>3.80</td>
<td>3.04</td>
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<tr>
<td>LC-37 femur</td>
<td>1.36</td>
<td>4.55</td>
<td>3.09</td>
<td>3.84</td>
<td>2.47</td>
</tr>
<tr>
<td>LC-38 femur</td>
<td>1.42</td>
<td>4.82</td>
<td>3.25</td>
<td>3.74</td>
<td>2.20</td>
</tr>
<tr>
<td>LC-39 femur</td>
<td>1.29</td>
<td>3.99</td>
<td>2.77</td>
<td>3.64</td>
<td>1.91</td>
</tr>
<tr>
<td>LC-40 femur</td>
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<td>4.31</td>
<td>2.85</td>
<td>3.77</td>
<td>2.09</td>
</tr>
<tr>
<td>LC-41 femur</td>
<td>1.31</td>
<td>4.30</td>
<td>3.07</td>
<td>3.55</td>
<td>2.11</td>
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</table>

<table>
<thead>
<tr>
<th>n</th>
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<th>11</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>1.32</td>
<td>4.44</td>
<td>3.06</td>
<td>3.52</td>
<td>2.30</td>
</tr>
<tr>
<td>SDEV</td>
<td>0.06</td>
<td>0.23</td>
<td>0.21</td>
<td>0.38</td>
<td>0.33</td>
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<tr>
<td>SEM</td>
<td>0.02</td>
<td>0.07</td>
<td>0.06</td>
<td>0.11</td>
<td>0.10</td>
</tr>
</tbody>
</table>

**Abbreviations:**
- M-L (medial-lateral)
- A-P (anterior-posterior)
- O.D. (outer diameter)
- I.D. (inner diameter)

### HLU Study #2: Group - ULS "Unloaded Synbiotic Diet"; i.e. weight OFF 2 hindlimbs; synbiotic diet feeding

**Rat Femur Bone Midshaft Diameter Measurements (Right Leg)**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Wet weight (g)</th>
<th>B (M-L) O.D. mm</th>
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<th>D (A-P) O.D. mm</th>
<th>d (A-P) I.D. mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>ULS-1 femur</td>
<td>1.34</td>
<td>4.86</td>
<td>2.89</td>
<td>3.93</td>
<td>1.89</td>
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<tr>
<td>ULS-2 femur</td>
<td>1.40</td>
<td>4.19</td>
<td>2.79</td>
<td>4.00</td>
<td>2.62</td>
</tr>
<tr>
<td>ULS-3 femur</td>
<td>1.29</td>
<td>4.53</td>
<td>2.72</td>
<td>3.88</td>
<td>2.22</td>
</tr>
<tr>
<td>ULS-5 femur</td>
<td>1.27</td>
<td>4.15</td>
<td>2.63</td>
<td>3.98</td>
<td>2.17</td>
</tr>
<tr>
<td>ULS-6 femur</td>
<td>1.34</td>
<td>4.63</td>
<td>3.17</td>
<td>3.79</td>
<td>2.43</td>
</tr>
<tr>
<td>ULS-7 femur</td>
<td>1.34</td>
<td>4.49</td>
<td>3.24</td>
<td>4.15</td>
<td>2.30</td>
</tr>
<tr>
<td>ULS-8 femur</td>
<td>1.22</td>
<td>4.36</td>
<td>2.68</td>
<td>3.77</td>
<td>2.16</td>
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<tr>
<td>ULS-9 femur</td>
<td>1.43</td>
<td>4.48</td>
<td>2.69</td>
<td>4.01</td>
<td>2.52</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>n</th>
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<th>8</th>
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<tr>
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<td>4.46</td>
<td>2.85</td>
<td>3.94</td>
<td>2.29</td>
</tr>
<tr>
<td>SDEV</td>
<td>0.07</td>
<td>0.23</td>
<td>0.23</td>
<td>0.12</td>
<td>0.23</td>
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<td>SEM</td>
<td>0.02</td>
<td>0.08</td>
<td>0.08</td>
<td>0.04</td>
<td>0.08</td>
</tr>
</tbody>
</table>
### HLU Study #2: Group - LS "Loaded Synbiotic Diet"; i.e. weight on all 4 limbs; synbiotic diet feeding

#### Rat Femur Bone Midshaft Diameter Measurements (Right Leg)

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<th>D (A-P) O.D. mm</th>
<th>d (A-P) I.D. mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS-21 femur</td>
<td>1.22</td>
<td>4.05</td>
<td>2.61</td>
<td>3.68</td>
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### HLU Study #2: Group - ULC "Unloaded Control"; i.e. weight OFF 2 hindlimbs; regular rat chow diet (no synbiotics)

#### Rat Femur Bone Midshaft Diameter Measurements (Right Leg)

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<th>b (M-L) I.D. mm</th>
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