Introduction
- Cleanrooms and environmental monitoring
- Contamination sources
- Contamination control
- The human microbiome and the microbial ecology of people
- Case study: Microorganisms are found in cleanrooms
- Why cleanroom microflora matters

Cleanrooms
- Cleanrooms: highly controlled environments with defined air quality
- Required for surgical units, hospital pharmacies and aseptic drug manufacture.
  - Airborne particulate control
  - Air movement and air direction,
  - HEPA filters,
  - Temperature and humidity control,
  - Cleaning and disinfection,
  - Staff behaviours and gowning.
- Certified to meet ISO 14644.
- Operated to meet EU GMP.

Contamination sources
- People
  - The body harbours over thirty million bacteria
  - The average person sheds 1,000,000,000 skin cells per day
  - 10% have micro-organisms on them
  - Particles are released from people via the mouth and nose (minute liquid droplets) – extremely contaminated with microorganisms.
Contamination sources

- **People**
  - The rate of particle deposition varies according to speed and activity.
  - When stationary, people generate approximately 100,000 particles of 0.3 µm or greater.
  - When walking slowly (2 miles per hour), people shed around 5 million particles.
  - When walking briskly (5 miles per hour), people shed around 7.5 million particles.

Contamination control #1

- Control:
  - Personnel wear suitable cleanroom clothing.
  - Good aseptic practices:
    - Slow, deliberate walking
    - Aseptic technique

Contamination control #2

- Air filtration
- UDAF devices
- Barrier technology: Isolators and RABS
- Water of low bioburden
- Keeping areas clean and dry
- Cleaning and disinfection
- Sterilisation
- Single-use disposable products

Environmental Monitoring

- Environmental monitoring is different to environmental control.
- Cleanrooms and clean areas are assessed through an environmental monitoring programme.
- Monitoring:
  - To assess cleanliness.
  - Physical assessment of airborne particles.
  - Checks on temperature, humidity, pressure etc.
  - Collection of data relating to the numbers and types of microorganisms present on surfaces, in the air and from people.

Microbiological environmental monitoring

- The classic techniques:
  - Active air-sampling: volumetric air-sampler
  - Passive air-sampling: settle plates
  - Surface samples: contact (RODAC) plates and swabs
  - Personnel samples: Finger plates and gown plates
- Methods highly variable.
- New generation of ‘real time’ viable air samplers.

Part 2: Microorganisms in cleanrooms
Microorganisms in cleanrooms

- Microbiologists count the number of ‘colony forming units’ (cfu) and identify the contaminants.
- To assess:
  - Cleanroom operation issues
  - Product risk investigations
  - Cleaning and disinfection issues.
  - Aseptic practices.

Microorganisms recovered

- Variables affect the types of microorganisms recovered:
  - Monitoring methods,
  - Location of the monitoring sample,
  - Types of agars,
  - Disinfectant neutralisers,
  - Incubation temperature,
  - Incubation time,
  - Identification method.

Part 3: The microbial ecology of people

- Many of the species of microorganisms that form the microbiota are unknown.
- Advanced through genotypic microbial identification techniques (16s rRNA genes in lysed microbial DNA).
- Now understood that:
  - Considerable diversity of species
  - Variation between different locations on the body
  - Variation between individuals
  - Our microbiome changes over time.

Human Microbiome Project

- The Human Microbiome Project (2008)
  - Dedicated to the types and variations of microorganisms in relation to people (human microbiome).
  - Microbiome: “the totality of microbes, their genetic elements (genomes), and environmental interactions in a particular environment”.
- The human body contains over 10 times more microbial cells than human cells:
  - $10^{12}$ bacteria on the skin,
  - $10^{10}$ in the mouth,
  - $10^{14}$ in the gastrointestinal tract
- Weighing 200g
Microbial ecology of people

Different sites on the body have different microbial communities. Based on nutritional and physicochemical requirements, Liebig’s law of the minimum states that microbial numbers are determined by the nutrients present. Shelford’s law of tolerance highlights non-nutritional factors governing growth, such as pH and temperature. Also, antimicrobial factors and mechanical removal factors, like skin shedding, play a role.

Age, host genotype, and gender all influence the microbial ecology of people. Males, for instance, have a high density of bacteria on their skin compared to females.

The human gastrointestinal tract is home to approximately 500 to 1000 species of bacteria. The oral cavity is a diverse, abundant, and complex microbial community. Bacteria accumulate on both the hard and soft oral tissues in biofilms.

Skin #1: The average adult has 1.8m² of skin. Regional variation includes:
- Temperature range 25-37°C
- Warmest area under the arms (36.6°C) and the coolest area the fingers (29.5°C).
- pH of the skin varies between acidic and neutral:
  - Forehead: pH 4.8 and under the arm: pH 6.9.
- Moisture: high moisture in areas like the axilla, groin, and areas between the toes, while other areas are relatively dry.

Skin #2: Cleanroom risk is a concern. With the skin epithelial cells continually being shed, many microbial communities on the external surface are unstable.
Microbial ecology of people

- Skin #3
  - Most relate to 4 phyla:
    - Actinobacteria: Gram-positive bacteria e.g. Micrococcus, Corynebacteria and Propionibacteria
    - Firmicutes: Gram-positive bacteria e.g. Clostridia and Bacillus
    - Proteobacteria: Gram-negative bacteria e.g. Escherichia, Salmonella, Vibrio, Helicobacter
    - Bacteroidetes: Other Gram-negative bacteria

Microbial ecology of people

- Skin #4
  - Why so many Gram-positive bacteria?
    - Dry environment with high osmotic pressure.
    - Gram-positive bacteria better adapted (resistant to desiccation).
    - However, certain occluded regions (such as toe webs), have more variation, including Gram-negative bacteria.

Microbial ecology of people

- Skin #5
  - So, different parts of the body lead to variation

<table>
<thead>
<tr>
<th>Region</th>
<th>Environment</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forehead &amp; scalp</td>
<td>Sebaceous glands</td>
<td>Dominated by propionibacteria</td>
</tr>
<tr>
<td>Toe webs</td>
<td>Ocluded: increased moisture</td>
<td>High microbial density:</td>
</tr>
<tr>
<td></td>
<td>and temperature</td>
<td>propionibacteria, lactobacilli,</td>
</tr>
<tr>
<td>Arm and legs</td>
<td>Dry</td>
<td>low microbial density:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>staphylococci and micrococci.</td>
</tr>
<tr>
<td>Sole of foot</td>
<td>High moisture due to shoes</td>
<td>Many staphylococci</td>
</tr>
<tr>
<td>Main body</td>
<td>Varied</td>
<td>Corynebacteria, fungi and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acinetobacter spp.</td>
</tr>
</tbody>
</table>

Microbial ecology of people

- Skin #6
  - Variation between people:
    - Ecologically, sebaceous areas had greater species richness than moist and dry one.
    - The areas with least similarity between people:
      - spaces between fingers, the spaces between toes, axillae, and umbilical cord stump.
    - The areas with most similarity between people:
      - beside the nostril, nares (inside the nostril), and on the back.

Microbial ecology of people

- Skin #7
  - The most frequent species are:
    - Bacteria:
      - Staphylococcus epidermidis, Streptococcus mitis, Propionibacterium acnes, Corynebacterium spp., and Acinetobacter johnsonii,
      - Some anaerobic bacteria, like Propionibacterium, in hair follicles.
    - Fungi:
      - Yeasts Candida albicans, Rhodotorula rubra, Torulaopsis, Microsporum gypseum, Trichophyton and fungi Rhizopus Trichosporon, Fusarium, Aspergillus and Penicillium species

Part 3: Why do microorganisms survive in cleanrooms?
Why do microorganisms survive in cleanrooms?

- Varies depending upon the species and different factors:
  - Temperature
  - Available water
  - Concentration of organic compounds
  - Concentration of hydrogen ions
  - Concentration of inorganic compounds
  - Concentration of particles in the air
  - Redox potential
  - Pressure
  - Light intensity
  - Geographical location and habitat

What is found in cleanrooms?

- Description of the human microbiota does not necessarily predict the types of microorganisms found in cleanrooms.
- Variations relating to:
  - Cleanroom types and uses
  - Grade, temperature and humidity
  - Geographical location
  - Gowning requirements
  - Certain locations of the body are more likely to release organisms than others.

What is found in cleanrooms?

- Variations also relate to:
  - Microbial identification method
    - Phenotypic or genotypic
  - Databases
  - Size, orientation (clinical or industrial)
  - Periodic reclassification of microorganisms
  - Limitations of EM methods
  - EM agar and incubation regime
  - Culturability
  - Some research suggest that less than 10% of bacteria found in cleanrooms are culturable
  - Seasonality and time of sampling

The study

- Very few studies of pharmaceutical cleanroom microflora published.
- A recent study is:


The study

- Pharmaceutical facility in south-east England
- The cleanrooms examined represented:
  - 40 Grade B rooms (of which five had Grade A cleanzones)
  - 35 Grade C cleanrooms
  - 20 Grade D cleanrooms
- EM regime used TSA and incubation regime:
  - 30-35°C ≤2 days and 20-25°C ≤5 days
- Time period: 2001 - 2010
The study

- EU GMP Grade A and B clean areas = 6,729 isolates

Order of detection:
- Micrococcus luteus
- Micrococcus lylae
- Staphylococcus spp
- Micrococcus spp
- Staphylococcus epidermidis
- Staphylococcus capitis
- Staphylococcus hominis
- Bacillus spp
- Staphylococcus haemolyticus

The study

- Grade A and B major species
- Skin related microflora represent the most common genera isolated, with the family Micrococcaceae (the genera Micrococi and Staphylococci) representing >90% of the isolates

Order of detection:
- Micrococcus luteus
- Micrococcus lylae
- Staphylococcus spp
- Micrococcus spp
- Staphylococcus epidermidis
- Staphylococcus capitis
- Staphylococcus hominis
- Bacillus spp
- Staphylococcus haemolyticus

The study

- EU GMP Grade C and D clean areas = 2,500 isolates
- More diversity due to nature of operations and presence of water

Order of detection:
- Micrococcus luteus
- Bacillus spp.
- Micrococcus lylae
- Micrococcus spp.
- Staphylococcus spp.
- Bacillus cereus
- Pseudomonads
- Corynebacterium spp

Outcomes

- Association between the microorganisms commonly found in cleanrooms and those which are transient to (short-term or long term-residents on) human skin.
- Low incidents of Bacillus spp.,
  - Possible transfer into the cleanrooms via personnel, dust, and material transfer.
- Occasional, low-level incidences of microorganisms resident within the human body.
- Where there is a water source, some microorganisms associated with water detected.

Outcomes

- Little variation over time
- Variation signalled that something had gone wrong e.g. cleaning techniques, changes to personnel, HVAC failure
- Sample types:
  - Personnel samples: Gram-positive cocci occur most frequently
  - Air samples: Gram-positive cocci occur very frequently (people shedding)
  - Surfaces: Gram-positive cocci occur quite frequently. Higher levels of Gram-positive rods (possible equipment transfer link)
  - Where Gram-negative rods occur, this is from water on surfaces

Outcomes

- Majority mesophilic aerobic or facultatively aerobic bacteria.
- Where specialist gases are used some anaerobic found.
- Thermophiles and extremotolerant bacteria very rare.
- Common fungi in cleanrooms are: Aspergillus, Penicillium and Trychophyton.
Reviewing microflora

- Understanding where contamination has come from
  - corrective and preventative actions
- Risk assessing possibility of microbial survival in non-sterile products
- Trending
  - Reviewing efficiency of cleaning and disinfection regimes e.g. are spore formers or Gram-negatives surviving?
- Culture media
  - Challenging the media with the most common isolates

Reviewing microflora

- Cannot identify everything.
  - Isolates over action level or 50% of all isolates.
- Is genus or species level needed?
  - Yes, if relating to product contamination
  - No, if carrying out general cleaning review

Summary

- Outlined the human microbiome
- Considered theoretical cleanroom microflora
- Microbial diversity observed in cleanrooms is much broader than previously realised.
- Knowing the normally recovered types of microorganisms:
  - understanding contamination origins provides information about:
    - people,
    - disinfection regimes,
    - air handling systems
    - cleanroom control.