Biofilm formation on dairy separation membranes as affected by the substrate and cheese starter

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By:
Ashraf Hassan
South Dakota State University

Other Researchers:

Dr. Sanjeev Anand
Nuria Garcia-Fernandez, PhD student

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Abstract
The objectives of the proposed project are: 1) to study the effect of feed (concentration; whey vs milk; pasteurized vs raw) on biofilm formation, and 2) to study the effect of cheese starter culture (ropy vs nonropy) on biofilm formation. A CDC bioreactor will be used to form biofilm in vitro using different feeds. The extent of biofilm formation as affected by the following factors will be evaluated: feed material (whey vs milk), the level of concentration (2 x, 4 x, and 8 x), heat treatment given to the feed (raw milk, pasteurized milk, and pasteurized whey), and starter culture (ropy cultures vs nonropy genetic variants). This research will provide useful information on factors affecting biofilm formation on dairy processing membranes and help develop effective cleaning protocols.

Background Information
Biofouling is one of the major operational problems in membrane filtration due to long periods between successive cleaning cycles (Muthukumaran et al., 2005). Biofilm may serve as a continuous contamination source of food spoilage bacteria and pathogens in the finished product (Herzberg and Elimelech, 2007; Tang et al., 2009). In a current research proposal, we observed extensive biofilm formation on whey RO and UF membranes (Hassan et al. 2010). Different factors affect biofilm formation on food contact surfaces. In the ongoing project, we study the role of bacterial cell and membrane surface characteristics in biofilm formation. Another important factor that may affect biofilm formation is the nature and composition of the substrate. Biofilms are multicellular communities encased in a self-produced polymeric matrix (EPS) (Chmielewski and Frank, 2003). Exopolysaccharides and their structure play a major role in formation and stability of biofilm. Our previous research showed that the amount of EPS and their structure are affected by the composition, pH, and osmolarity of the growth medium (Hassan et al. 2001; Hassan, 2008). Therefore, we hypothesize that the extent of biofilm and its structure would be affected by the growth substrate and nutrients availability. Whey and milk concentration and fractionation are common in today's dairy industry. Our current focus is on biofilm formation on whey RO membranes. Biofilm formation on whey RO and milk UF membranes is expected to be different due to the following reasons: 1) the type and number microorganisms in the feed of the two systems are different, 2) the composition of the feed is different which would affect not only the microbial growth and amount and structure of EPS they produce but also biofilm formation, structure and stability, and 3) the structure of RO and UF membranes are different.

During membrane separation, a concentrated solute is built up at the membrane surface. This leads to concentration gradient of solute from the membrane to the bulk stream, a phenomenon called concentration polarization. Therefore, microorganisms involved in biofilm formation grow in a medium that is different from both the feed and the final product. It is very important to understand the effect of that medium on biofilm formation. This will help industry develop effective cleaning and sanitation regimes and decide on cleaning frequency.

Another important factor that may contribute to biofilm formation is the application of EPS-producing cultures in making cheese. Such cultures are commonly used to improve texture of reduced fat cheeses (Hassan et al. 2005; Hassan, 2008). Whey from cheese making would contain a high count of these cultures. In addition to their ability to produce biofilm, EPS-producing starters may also support biofilm formation by non-EPS-producing cultures or harbor them (Hassan et al. 2004).

Objectives

Objective 1: To study the effect of cheese starter culture (ropy vs non-ropy) on attachment and biofilm formation on dairy filtration membranes.

Objective 2: To study the effect of feed (M17 vs concentrated whey) on biofilm formation by cheese starters.

Bacterial cultures:

To evaluate the effect of cheese starter (ropy vs non-ropy) on biofilm formation. Two ropy cultures (*Streptococcus thermophilus* 3534 and *Lactococcus lactis* ssp. *cremoris* JFR) and their EPS-negative genetic variants (*Streptococcus thermophilus* 5842 and *Lactococcus lactis* subsp *cremoris* JFR-1 respectively) were used to form biofilm on dairy filtration membranes. The ropy *Streptococcus thermophilus* 3534 and its non-ropy variant 5842 obtained by spontaneous mutation were provided by Chr. Hansen. The ropy *Lactococcus lactis* ssp. *cremoris* JFR producing high amounts of extracellular polysaccharides (slime) was isolated from a retail dairy product and its non-ropy mutant was obtained in our lab by spontaneous mutation. Interestingly, the mutant lacked the genes for EPS production and lactose fermentation so we substituted lactose in the growth medium with glucose. Growth curve analysis was done for the two EPS-producing cheese starters and their respective non-EPS producing mutants before conducting biofilm experiments to rule out any growth differences between the genetic variants.

Membrane preparation:

Reverse osmosis (RO) membranes were used in our experiments. In order to allow bacteria to grow and form biofilm on only the retentate side of the membranes, two membrane pieces were cut and glued together using water resistant glue which exposed only the retentate side. This allowed us to use the stomacher not a subjective method like swabbing to detach cells. Four cm^2 pieces of the glued membranes were cut and used in the biofilm experiments.

Five pieces (2x2 cm) of membranes were used in each treatment. They were cut under aseptic conditions and pretreated by soaking for 5 days in sterile distilled water as suggested by the manufacturer to get rid of the organic materials covering new membranes. Membrane pieces were disinfected in 0.5% solution of H_2O_2 followed by two rinsing steps in sterile distilled water for 15 minutes to remove the sanitizer residues. Membranes were kept in 150 x 15 mm petri dishes (5 per plate).

Medium preparation:

M17 broth supplemented with filter sterilized 0.5 or 5% lactose, or 2.5% glucose solutions, and 10% solution of whey protein concentrate 35 (WPC 35) sterilized by filtration were used as the growth media.

Inoculum preparation:

Single colonies from pure culture plates of EPS+ and EPS- *Streptococcus thermophilus* or EPS+ and EPS- *Lactococcus lactis* ssp. *cremoris* were separately activated and propagated in 50 ml of M17 broth incubated at 30°C and 35°C respectively under aerobic conditions.

Attachment of bacteria to RO membranes in the absence of growth:
Active bacterial cultures (10^8 cfu/ml) grown in M17 broth at their late logarithmic phase were washed once in sterile phosphate buffer saline, suspended in M17, and added to petri dishes containing 5 pieces of the membranes prepared as described above. Dishes were incubated for 24 hours at 4°C.

Biofilm formation on RO membranes:
The following media were used in the biofilm experiments: 1) M17 broth containing 0.5% lactose, 2) M17 broth containing 5% lactose, M17 containing 2.5% glucose, and 3) 10% WPC 35. Fifty ml of the culture was poured onto a petri dish containing 5 pieces of the membrane prepared as described above. Plates were incubated for 24 hours at 30 °C (for *Lactococcus*) or 35 °C (for *Streptococcus*).

Assessment of cells attached to RO membranes:
To determine the number of attached microorganisms, membranes were rinsed 3 times/30 sec each in sterile H_2O to remove loosely attached cells, placed in sterile plastic bags containing 99 ml of PBS, and stomached at 230 rpm for 2 minutes to detach cells. Samples were serially diluted in PBS and then 0.1 ml of each dilution was pipetted and spread on the surface of M17 agar plates. Plates were incubated overnight at 30 °C (*Lactococcus*) or 35°C (*Streptococcus)* and counts were expressed as cfu/cm^2. Each experiment was replicated 3 times.

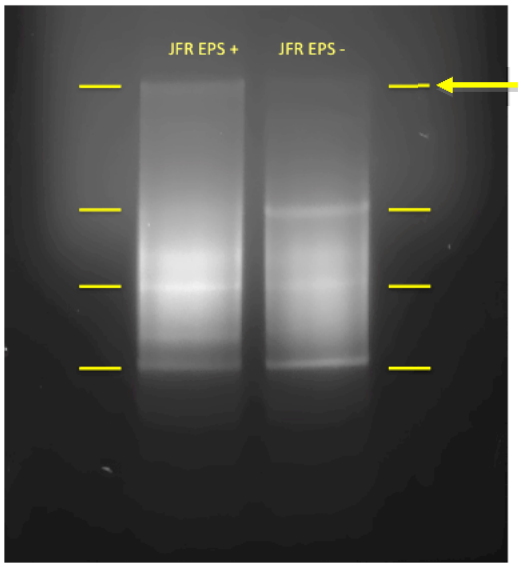
Statistical analysis:
Statistical analysis was completed using SAS 9.3 (TS LEVEL 1M0 W32_7PRO platform, Cary, NC (USA). The pair-wise comparisons between treatments using a Student's T-test were performed to examine significant differences among treatments. The significance level was set at 5%.

Results and Discussion

Selection of a *Lactococcus lactis ssp. cremoris* mutant lacking EPS production:
Since the genes for EPS production in *L. lactis ssp. cremoris* strain JFR are plasmid encoded, we were able to obtain an EPS-negative variant by spontaneous mutation. The EPS-negative mutant lost the ability to ferment lactose, so it is likely that both genes are encoded in the same plasmid.

The mutant deriving from *L. lactis ssp. cremoris* was Gram-positive cocci, catalase negative and grew at the same rate as the parent strain when glucose was added to the growth medium. Plasmid DNA was extracted (Miniprep, QIagen) and separated in agarose gel electrophoresis (0.7% agarose) using TAE buffer for 90 minutes at 80 mvol. The plasmid profile comparison revealed the loss of a plasmid band on the top of the profile of JFR EPS^-.

Figure 1. Plasmid profiles of *L. lactus spp. cremoris* JFR EPS+ and its spontaneous non-ropy mutant. The arrow indicates the plasmid absent from the nonropy mutant.



Attachment of *Streptococcus thermophiles* to RO membranes in the absence of growth:
To study the role of capsular EPS of *Streptococcus thermophilus* in attachment to dairy separation membranes, the EPS+ and EPS- genetic variants of *S. thermophilus* were incubated with pieces of the membrane at 4 ºC for 24h. Tables 1 shows no significant differences ($P > 0.05$) in attachment between the genetic pair.

Table 1. Counts (log cfu/cm) of ropy and non-ropy *S. thermophilus* attached to RO membranes at 4 ^{o}C. Data are the average of 3 replicates (n=15).

Treatment	Log CFU	Std Dev	Std Err
EPS+	5.9176	0.5978	0.1543
EPS-	6.1228	0.4956	0.1280
Diff (1-2)	-0.2052	0.5491	0.2005

Data in the same column with no superscript are not significantly different ($P > 0.05$).

Attachment of *Lactococcus lactis* subsp *cremoris* on RO membranes in the absence of growth:
Table 2 shows attachment of the genetic pair of *L. lactis* ssp. *cremoris* to membranes in the absence of growth. Since the EPS- variant did not grow on lactose, glucose was used at 2.5% to give the same osmolality produced by 5% lactose in the EPS+ culture medium.

A significantly lower number of the ropy strain of *L. lactis* ssp. *cremoris* attached to the membrane than that of the EPS- genetic variant (Tables 3), suggesting that EPS produced by this strain interfered with the adhesion to the membrane surface.

Table 2. Counts (log cfu/cm^2) of cells of ropy and non-ropy *L. lactis* subsp *cremoris* attached to the RO membrane. Data are the average of 3 replicates (n=15).

Treatment	Log CFU	Std Dev	Std Err
EPS+	4.7848^b	0.2070	0.0534
EPS-	6.1625^a	0.2151	0.0555
Diff (1-2)	-1.3777	0.2111	0.0771

Data in the same column with no common superscript are significantly different ($P < 0.05$).

Biofilm formation by *Streptococcus thermophilus* on RO membranes:

a. Biofilm formation in M17 broth containing 0.5% lactose:

Table 3 shows that counts of cells in the biofilm formed by the EPS+ strain *S. thermophilus* were significantly ($P < 0.05$) higher than those of the EPS- variant.

Table 3. Counts (cfu/cm^2) of ropy and non-ropy *S. thermophilus* in biofilm formed on RO membranes using M17 containing 0.5% lactose. Data are the average of 3 replicates (n=15).

Treatment	Log CFU	Std Dev	Std Err
EPS+	6.7610[a]	0.2159	0.0557
EPS-	4.0136[b]	0.2399	0.0620
Diff (1-2)	2.7473	0.2282	0.0833

Data in the same column with no comment superscript are significantly different ($P < 0.05$).

Biofilm formation in M17 containing 5% lactose:

Table 4 shows that counts of cells in the biofilm formed in M17 containing 5% lactose by the EPS+ strain *S. thermophilus* were significantly (P< 0.05) higher than those of the EPS- variant. However, there were less differences between the genetic pair in M17 containing 5% lactose than those in M17 containing only 0.5% lactose (Table 3) as the EPS- counts were higher in 5% lactose than in 0.5% medium whilst the EPS+ counts were the same in both media.

Table 4. Counts of ropy and non-ropy *S. thermophilus* in biofilm formed on RO membranes (log cfu/cm) using M17 with 5% lactose. Data are the average of 3 replicates (n=15).

Treatment	Log CFU	Std Dev	Std Err
EPS+	6.4613[a]	0.1318	0.0340
EPS-	5.4997[b]	0.3271	0.0844
Diff (1-2)	0.9616	0.2493	0.0910

Data in the same column with no common superscript are significantly different (*P < 0.05*).

Biofilm formation in 10% WPC 35:

Table 5 shows that counts of the EPS+ *S. thermophilus* cells in the biofilm formed on RO membranes using 10% WPC 35 as a growth medium were significantly (P< 0.05) higher than those of the EPS- variant.

Table 5. Counts of ropy and non-ropy *S. thermophilus* in biofilm formed on RO membranes (log cfu/cm) using
10% WPC 35 as a growth medium. Data presented the average of 3 replicates (n=15).

Treatment	Log CFU	Std Dev	Std Err
EPS+	5.9567[a]	0.2508	0.0648
EPS-	5.2171[b]	0.3045	0.0786
Diff (1-2)	0.7396	0.2789	0.1019

Data in the same column with no common superscript are significantly different (*P < 0.05*).

Biofilm formation by *Lactococcus lactis* ssp. *cremoris* on RO membranes:

a. Biofilm formation in M17 containing 0.5% lactose or 2.5% glucose:

Table 6 shows that counts of cells of EPS+ and EPS- strains of *L. lactis* ssp. *cremoris* in the biofilm formed on RO membranes using M17 containing 0.5% lactose (for EPS +) and 2.5% glucose (for EPS -) were not significantly different *(P> 0.05)*.

Table 6. Counts (cfu/cm^2) of ropy and non-ropy *L. lactis* subsp *cremoris* in biofilm formed on RO membranes using M17 containing 0.5% lactose. Data are the average of 3 replicates (n=15).

Treatment	Log CFU	Std Dev	Std Err
EPS+	6.7687	0.3289	0.0849
EPS-	6.9871	0.3135	0.0810
Diff (1-2)	-0.2183	0.3213	0.1173

Data in the same column with no superscript are not significantly different (*P > 0.05*).

b. Biofilm formation in M17 containing 5% lactose or 2.5% glucose:

Table 7 shows that counts of cells in the biofilm formed in M17 containing 5% lactose by the EPS+ strain *L. lactis* ssp. *cremoris* were significantly (P< 0.05) lower than those of the EPS- variant (grown in the presence of 2.5% glucose). There were more differences between the genetic pair in M17 containing 5% lactose than in M17 containing 0.5% lactose. This observation suggests that presence of higher amounts of the precursors for EPS production originated from the galactose moiety of lactose seemed to influence biofilm formation on RO membranes as EPS produced by this strain interfere with the interactions that occur between the bacterial cells and surface.

Table 7. Counts (cfu/cm^2) of ropy and non-ropy *L. lactis* subsp *cremoris* in biofilm formed on RO membranes using M17 containing 5% lactose. Data are the average of 3 replicates (n=15).

Treatment	Log CFU	Std Dev	Std Err
EPS+	4.5543^{b}	0.2865	0.0740
EPS-	6.0230^{a}	0.4446	0.1148
Diff (1-2)	-1.4687	0.3740	0.1366

Data in the same column with no common superscript are significantly different (*P < 0.05*).

Biofilm formation in 10% WPC 35:

Table 8 shows that counts of cells in the biofilm formed in 10% WPC 35 by the EPS+ strain *L. lactis* ssp. *cremoris* were significantly (P< 0.05) lower than those of the EPS- variant. The biofilm formation in 10% WPC 35% was lower than that in M17. This was consistent with the findings of the *S. thermophilus* experiments.

Table 8. Counts of ropy and non-ropy *L. lactis* subsp *cremoris* in biofilm formed on RO membranes (log cfu/cm) using 10% WPC 35 as a growth medium. Data are the average of 3 replicates (n=15).

Treatment	Log CFU	Std Dev	Std Err
EPS+	4.3305[b]	0.3224	0.0832
EPS-	5.6677[a]	0.5955	0.1538
Diff (1-2)	-1.3372	0.4789	0.1749

Data in the same column with no common superscript are significantly different (*P < 0.05*).

Conclusions

While EPS produced by *S. thermophilus* strongly supported biofilm on RO membranes, EPS produced by *L. lactis ssp. cremoris* seemed to interfere with the initial phase of biofilm formation (adhesion). Differences in the molecular characteristics of EPS and their interaction with the membrane surface may explain these observations. Sugar composition, branching, charge, and hydrophobicity are some possible factors influencing the behavior of EPS and their role in bacterial attachment to surfaces like dairy separation membranes.

We obtained a mutant of *L. lactis ssp. cremoris* that does not grow in the presence of lactose as a sole carbon source. We propose that EPS-production and lactose fermentation genes could be encoded on the same plasmid, although this needs to be confirmed by additional studies. The difference in adhesion and biofilm formation between the *L. lactis ssp. cremoris* genetic variants could be related to only EPS production since it's unlikely that the EPS- mutant lost genes encoding adhesion-related proteins along with the plasmid that carried EPS synthesis and lactose fermentation genes.

The findings of this research indicate that the type of starter culture used in cheese making may impact biofilm formation on dairy filtration membranes. EPS production may enhance or prevent biofilm formation depending on their type. Future analysis will be done in our laboratory to determine differences in molecular characteristics of EPS produced by *S. thermophilus* and *L. lactis ssp. cremoris*. This will help us understand the mechanism by which EPS enhance or inhibit biofilm formation.

Substrate used to form biofilm did not affect the tendency of the two cheese starters to form biofilm. However, the counts of attached cells grown on M17 were greater than those on WPC.

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